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Modified neutral comet assay for human lymphocytes

Maria Wojewódzka,
Iwona Grądzka,
Iwona Buraczewska

Abstract Comet assay under neutral conditions allows the detection of DNA double-strand breaks, considered to be the biologically relevant radiation-induced lesion. In this report we describe modifications of the neutral comet method, which simplify and facilitate its use for estimation of DNA double strand breaks in human lymphocytes irradiated with doses of ^{60}Co gamma-rays (from 10 to 100 Gy). The analysis carried out according to this protocol takes less time than those published so far. Also, the use of lysis at 50°C is avoided; this is important in view of the presence of heat-labile sites in the DNA of irradiated cells, recently reported by Rydberg [12]. The comets have well defined, sharp limits, are suitable for computer image analysis and chromatin of the control cells remains condensed.

Key words DNA double strand breaks • human lymphocyte • ionising radiation • neutral comet assay

Introduction

The single-cell gel electrophoresis or comet assay is a sensitive fluorescence microscopic method that has found numerous applications. Among others, it is used for DNA damage detection for radiobiological and toxicological purposes, in biomonitoring, identification of irradiated food etc. (for reviews, see [2–4, 6]). The method allows to detect DNA damage in individual cells and to estimate its distribution in cell populations. Further advantages are a considerable sensitivity, especially under alkaline conditions and the possibility of application of endonucleases or antibodies that recognise specific damage types.

More than 20 years ago Rydberg and Johanson [13] observed relaxation of chromatin from irradiated cells under conditions where control chromatin remained condensed. In the initial version of the comet assay Östling and Johanson [10] applied electrophoresis under neutral conditions, whereas later, Singh *et al.* [14] used alkaline pH; this allowed to increase the sensitivity of the assay, since some DNA unwinding could take place during lysis and electrophoresis. Later studies [17–19] further improved the method, which could detect damage induced by mGy doses [18]. It became a popular way of measuring the sum of single and double strand breaks (SSB and DSB, respectively) generated by various DNA damaging agents.

The comet assay was further modified for estimation of DSB, by using an electrophoresis buffer at non-denaturing pH and various lysing conditions in the so-called neutral version of the assay. In cells damaged by ionising radiation DSB are about 25 times less numerous than SSB. To enhance the sensitivity of the neutral assay, Olive *et al.*

M. Wojewódzka✉, I. Grądzka, I. Buraczewska
Department of Radiobiology and Health Protection,
Institute of Nuclear Chemistry and Technology,
16 Dorodna Str., 03-195 Warsaw, Poland,
Tel.: +48 22/ 811 07 36; Fax: +48 22/ 811 15 32,
e-mail: marylaw@orange.ichtj.waw.pl

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[1, 5, 7–9, 17, 19] increased the time of lysis to 4–5 h and the temperature of lysis to 40–50°C. Under these conditions the radiation dose – tail moment relationship was linear in the dose range from 5 to 300 Gy. The sensitivity could further be increased by incubation of the lysed cells in RN-ase and proteinase K [15, 16].

The neutral version of the method needs adaptation for the individual cell types to achieve optimal sensitivity. Here, we describe a modification of the neutral comet assay for human lymphocytes, which gives a linear radiation dose – tail moment relationship in the dose range from 10 to 100 Gy. The analysis carried out according to this protocol is less time-consuming than those published so far. Also, the use of lysis at 50°C is avoided; this is important in view of the presence of heat-labile site in the chromatin of irradiated cells, as reported by Rydberg [12]. The comets have well defined, sharp limits, suitable for image analysis and chromatin of the control cells remains condensed.

Materials and methods

Examined subjects and cell preparation

Five male blood donors were volunteers (non-smokers, mean age ± standard deviation (SD) = 24.8±3.5), informed about the aim of the study and experimental details. Blood was obtained by venopuncture; the lymphocytes were isolated with the use of Histopaque (Sigma) according to the procedure indicated by the producer. Immediately after isolation, the cells were suspended in phosphate buffered saline (PBS) and placed in an ice-water bath, or further incubated in RPMI medium with 20% foetal calf serum at 37°C in a CO_2 incubator. The whole procedure was carried out in a darkroom under red light. All chemicals including media and serum were purchased from Sigma, unless otherwise indicated.

Before gamma-irradiation, one volume of the lymphocyte suspension in PBS was mixed with three volumes of 1% solution of low melting point agarose (Type VII) in PBS at 37°C and immediately placed on microscopic slides under coverslips for 2–3 min on an ice-cooled metal plate. The microscopic slides were pre-coated with 0.5% regular agarose (Type I-A). After agarose solidification the slides were gamma-irradiated or sham irradiated.

Irradiation

Irradiation was carried out with ^{60}Co gamma-rays (Issledovatel) at a dose rate of about 3.28 kGy/h. Irradiation for initial damage determination was carried out at 0°C. For repair determination, the cells were placed in RPMI medium with 20% foetal calf serum, gamma-irradiated at 37°C with 10 Gy and further incubated for repair intervals of 15, 30, 60 and 120 min.

Lysis conditions

The coverslips were removed and the slides placed in the lysing solution. The basic buffer was composed of 2.5 M NaCl, 100 mM EDTA, 10 mM Tris HCl, 1% N-lauroylsarcosine, pH 9.5; it was prepared earlier and kept at room temperature for up to 1 month. Immediately before use, we added to this buffer 0.5% Triton X-100, 10% dimethylsulphoxide (DMSO), mixed for about 20 min and cooled in a refrigerator. In thus prepared lysing solution, the slides were left at 4°C, in the dark for 1–2 h, then washed 3 times with the electrophoresis buffer (300 mM sodium acetate, 100 mM Tris HCl, pH 8.5) and left in a fresh portion of the buffer for at least 1 h.

Electrophoresis

After lysis the slides were placed in a horizontal gel electrophoresis chamber filled with a fresh electrophoretic buffer. After electrophoresis (1 h at 14 V (0.5 V/cm) and 11–12 mA and temperature not higher than 10°C) the slides were washed with 0.4 M Tris, pH 7.5 (3×5 min) and then stained with 1 μM 4',6-diamidine-2-phenylindole dihydrochloride (DAPI, 50 μl) and covered with cover glasses. The microscopic slides were stored on a wet filter paper in air- and light-tight boxes in refrigerator for about 20 h. Thus prepared, the slides were ready for analysis.

Image analysis

Pictures of 100 randomly selected comets per slide for each donor in 3 separate experiments were captured at 200× magnification using a fluorescence microscope (Labophot-2, Nikon) equipped with a UV-1A filter block

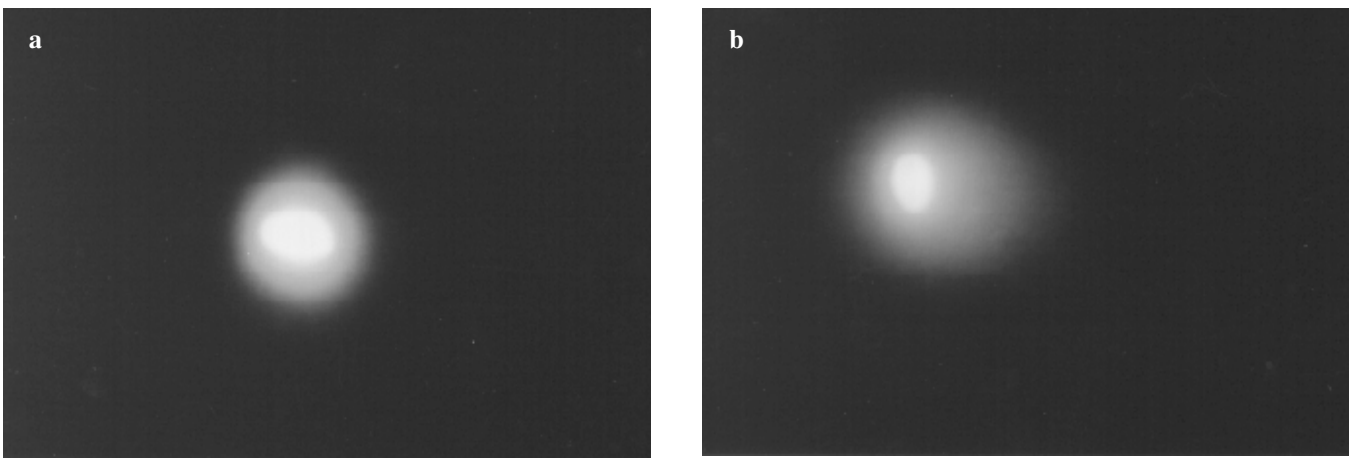

Fig. 1. Comets from human lymphocytes as seen in a fluorescent microscope (200× magnification) after lysis and electrophoresis in non-denaturing pH; a – control, b – gamma-irradiated with 10 Gy.

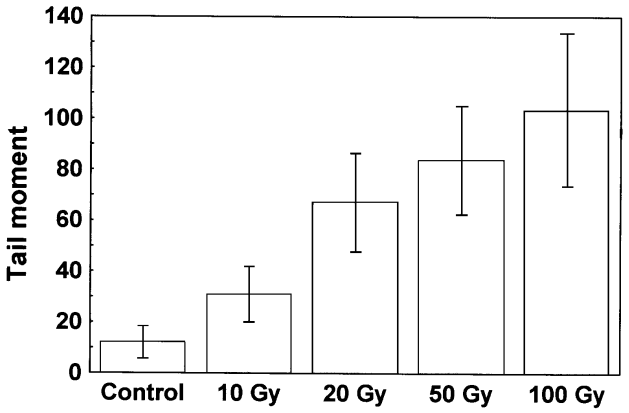

Fig. 2. Initial DNA damage measured by the neutral comet assay in gamma-irradiated in vitro human lymphocytes (from one donor). Mean tail moment from 100 comets per dose, SD indicated.

(an excitation filter of 365/10 nm and a barrier filter of 435 nm). Image analysis of data was by the Comet v.3.1 (Kinetic Imaging Ltd., Liverpool, UK). The measure of damage was tail moment (percentage of DNA in the tail times tail length). Data analysis was based on mean population response or on the distribution of damage among cells. In repair experiments, 50 comets per point were analysed. Mean values and SD are presented in the Figures. Statistical evaluation and plots were prepared with Statistica 5.1 software (StatSoft, Inc Tulsa, USA).

Results and discussion

Before introducing the described modifications, we applied for human lymphocytes the procedures published previously [1, 5, 7–9, 15, 17, 19] and obtained comets with very long and ill-defined tails, not suitable for computer image analysis. With the modified procedure the comets had well defined limits and their parameters could be accurately measured. Fig. 1 shows examples of the control (a) and irradiated (b) comets.

In comparison with the results of other authors [1, 5, 7–9, 17, 19] our modification did not lower the sensitivity of the comet assay: Fig. 2 presents the mean tail moments measured in lymphocytes from one donor after irradiation with doses from 10 to 100 Gy. The difference between the tail moments for 0 and 10 Gy samples is statistically significant (Student's test, $P<0.001$).

Fig. 3 shows the initial DNA damage distribution expressed as tail moment. A similar distribution was reported by Banath *et al.* [1] using neutral comet assay with 2.5 h lysis at 50°C. There was no subpopulation of cells differing in the amount of radiation induced DNA damage. Further, DSB repair was analysed in lymphocytes from 5 donors after irradiation with 10 Gy and expressed in absolute values of the tail moment (Fig. 4a) or as % of the initial damage (Fig. 4b). In spite of inter-individual differences (Fig. 4a), the repair course is fairly similar when relative values are plotted. The repair curves show a typical biphasic shape. The level of the residual DSB after 2 hours repair, expressed as % of the initial damage, is in the range from 14.4% to 24.5%. Using as an example the data obtained for donor 2 (Fig. 5), it is shown that the repair can be described by the equation

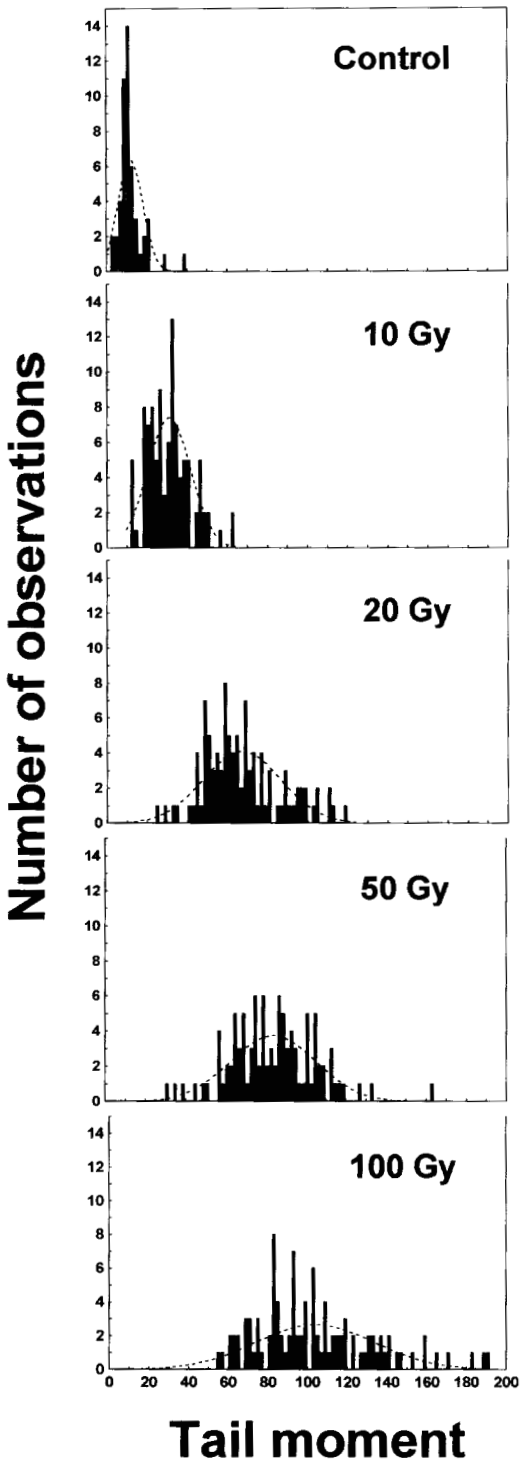

Fig. 3. Distributions of DNA damage for the same set of result as shown in Fig. 2.

$$y = a^{*}\exp(-b\tau)+c \quad (1)$$

where c is the residual unrepairable damage, a is the initial repairable damage (thus, total initial damage equals to $a+c$), and $b(-1/\tau)$ is the repair coefficient (τ is time necessary to reduce the damage to 37% of the initial level). Fig. 6 shows the change in distribution of DNA damage during repair shown for the same set of results as those in Fig. 4. Rydberg [12] suggests that the fast repair phase correspond to the repair of lesions converted to

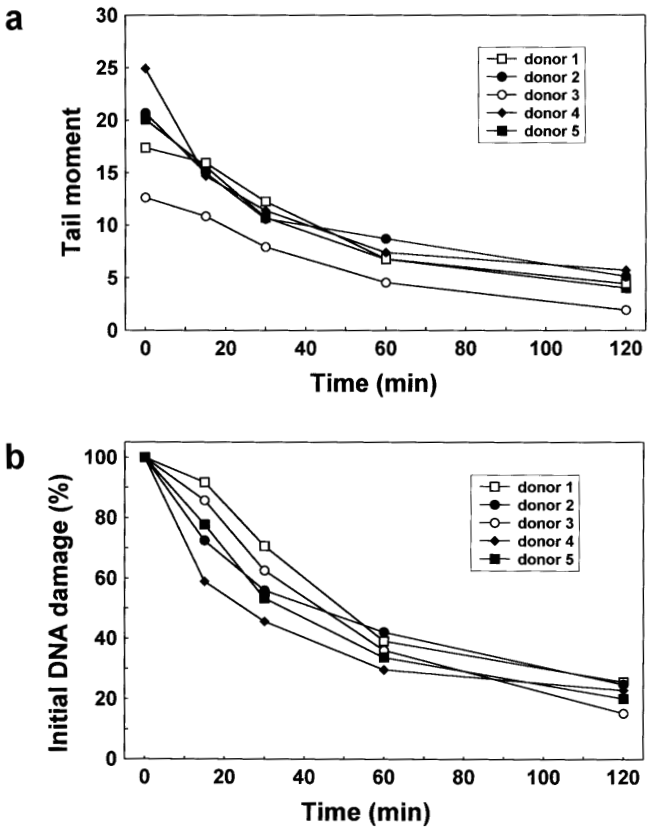

Fig. 4. Repair of DSB produced by 10 Gy of gamma-rays in isolated lymphocytes from 5 donors; a – individual repair curves with DNA damage expressed as mean tail moment; b – the same results expressed as % of the initial damage. For each time point, the mean tail moment of the control was subtracted from that measured for the irradiated cell population.

DSB by heat, not to the "true" DSB. The presented repair curves (Fig. 5) are biphasic, in spite of elimination of the lysis step at 50°C in our procedure; however, the part corresponding to the fast phase of repair is relatively shorter than most repair curves obtained with methods where the lysis at about 50°C is applied. The contribution of heat-labile lesions to the measured DSB yield can be considerable. Our (unpublished) results of experiments with Chinese hamster ovary cells show that the apparent increase in DSB resulting from lysis at 43°C is by a factor of about 4.5.

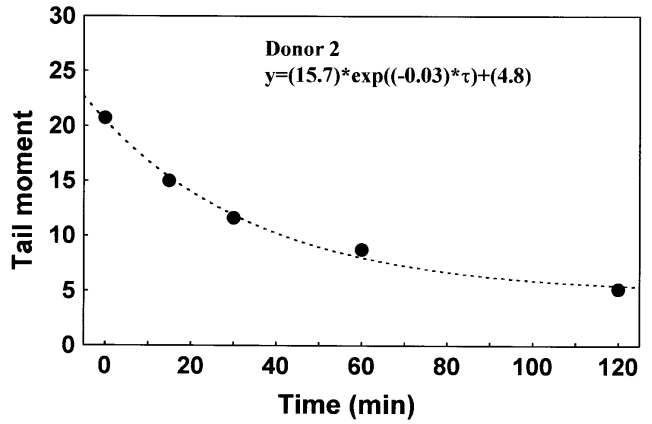

Fig. 5. Repair of DSB produced by 10 Gy of gamma-rays in isolated lymphocytes from donor 2; the fit for equation $y=a^*\exp(-b\tau)+c$ (see text) is shown; regression coefficient is 0.99.

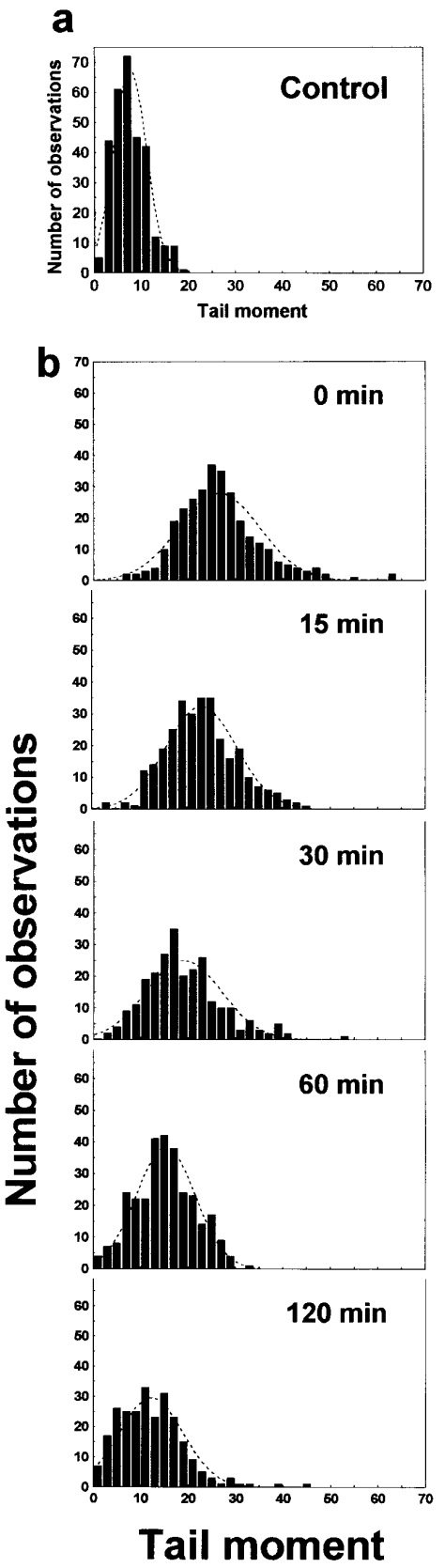

Fig. 6. The change in distribution of DNA damage during repair shown for the same set of results as that in Fig. 4. a – the control distribution, b – distribution of mean tail moment for 5 donors.

Altogether, the presented plots indicate that with the applied modification of the neutral comet assay, 10 Gy is an adequate dose for studying DSB repair by the neutral comet assay. Since usually several tens of Gy are used for studies of this kind, this is a step towards the clinically use-

ful predictive test that would be easier to perform than determination of SF2. Survival after irradiation with 2 Gy (SF2) is at present the most reliable indicator of intrinsic radiation sensitivity and finding a less time-consuming and equally reliable measure, well correlated with SF2 would be desirable (see Price *et al.* [11] for discussion).

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