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Calibration of the comet assay for the measurement of DNA damage in mammalian cells

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Abstract

We used X-rays from a linear accelerator and from a low energy therapeutic source to calibrate the single cell gel electrophoresis (comet assay), a widely used method to measure DNA damage. γ-Rays from ^{60}Co, with known efficiency in inducing DNA breakage, were used as reference. Human lymphocytes and one murine tumour cell line, F10-M3 cells, were irradiated under different experimental conditions. A similar relationship between radiation dose and induced DNA damage was obtained with γ- and X-rays. A calibration curve was constructed to convert the comet assay raw data into break frequency. The median levels of DNA breaks and oxidative damage in circulating lymphocytes from healthy volunteers were calculated to be 0.76 and 0.80 breaks/10^9 Da, respectively, (0.50 and 0.52 breaks/10^6 bp). The values of oxidative DNA damage were in the same order of magnitude as those found by others with HPLC methods.

Keywords: *Oxidative DNA damage, radiation damage, comet assay, human lymphocytes*

Introduction

Oxidative DNA damage is thought to play a role in the development of some degenerative diseases including cancer [1]. Thus, the measurement of DNA oxidation in human tissues has been considered a relevant endpoint both in observational and intervention epidemiological studies [2,3]. Peripheral white blood cells (WBC) have often been used in human studies both for their easy accessibility and on the assumption that oxidative DNA damage in WBC can reflect the level of oxidative stress in the body.

Therefore, much effort has been devoted to optimising methods for detection of oxidative DNA damage [4,5]. Among these, the most widely used in a variety of pathological conditions are the HPLC measurement of selected oxidised bases, such as 8-oxo-7,8-dihydroguanine (8-oxoGua), and the single cell gel electrophoresis or "comet assay" [6,7].

The comet assay is an electrophoretic procedure designed to measure DNA strand breaks (SBs) indirectly with high sensitivity, based on the extent of DNA migration at the single cell level in a electric field [8]. Specific oxidative damage can also be measured with the comet assay [9], using repair endonucleases, such as bacterial formamidopyrimidine DNA glycosylase (FPG), which recognises oxidised purines and formamidopyrimidine lesions and introduces further breaks at these sites.

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The oxidation damage detected by means of enzymatic digestion is usually referred to as "FPG-sensitive sites". Other enzyme-based methods for the detection of oxidised bases are alkaline elution [10] and alkaline unwinding [11].

In general, the HPLC methods are less sensitive than the enzyme-based ones and require relatively large amounts of DNA. Furthermore, it was reported that these procedures can induce the formation of artefactual oxidation damage, particularly during DNA isolation and hydrolysis [10]. However, HPLC methods provide a direct measurement of oxidised bases, whereas with the enzymatic methods only indirect quantification of the enzyme-induced breaks can be obtained. Thus, in order for the enzymatic methods results to be comparable with those obtained with chromatography, it is necessary to calculate the actual number of DNA breaks in comet assay experiments by calibration with a standard procedure of known efficiency in inducing DNA breakage, such as irradiation with γ-rays from ^{60}Co [12]. Calibration of the comet assay with X-rays has also been reported [13,14]. However, the energy and the biological efficacy of X-rays can vary considerably with the type of source. Thus, in order to use X-rays for calibration, it is necessary to select a type of X-radiation with the same biological efficacy than the reference radiation, i.e. γ-rays from ^{60}Co.

In the present study we used the comet assay with FPG to measure both the basal level of DNA SBs and oxidised purines in a sample of lymphocytes from 70 healthy volunteers. Calibration was performed using γ-rays from ^{60}Co, X-rays from a linear accelerator, and low energy X-rays from an apparatus used for Roentgen-therapy.

Materials and methods

Subjects

The subjects ($n = 70$, 44 females and 26 males, mean age 46.6 ± 1.86 years) were healthy volunteers recruited in previous studies. The mean body mass index (BMI) was 24.7 ± 0.44 kg/m^2 (mean ± standard error (SE)). These studies were conducted according to the principles of the Helsinki declaration, and were approved by the local Ethical Committee.

Cell cultures

F10-M3 cells, a clone isolated from the murine B16-F10 melanoma cell line, were kindly supplied by Professor Calorini, Department of Experimental Pathology and Oncology, University of Florence. The F10-M3 cells were grown in Dulbecco's Modified Eagle Medium containing 4500 mg/ml glucose (DMEM 4500) (Gibco, Paisley, UK), supplemented with 10% foetal calf serum (Invitrogen, San Giuliano Milanese, Italy) at 37°C in a 10% CO_2-humidified atmosphere.

To obtain a free cell suspension, the cells attached to a culture flask were trypsinised on ice, centrifuged for 5 min at 4°C, 250*g* and resuspended in fresh complete medium.

NCI-H520 (American Type Culture Collection, Rockville, MD, USA) cells were established from a human squamous cell carcinoma cell line and were grown attached to the bottom of a culture vessel as a monolayer, with RPMI 1640 medium (Sigma) supplemented with 5% foetal calf serum (FCS, Sigma), 2% HEPES 1 M (Sigma), 1% sodium pyruvate (Sigma) and 1% Pen-Strep (10,000 U Pen/ml–10,000 μg Strep/ml, Sigma). Cells were incubated at 37°C in an atmosphere with 95% relative humidity and 5% CO_2.

Freshly isolated human lymphocytes

Lymphocytes were isolated from peripheral blood samples of volunteers utilising the standard method of centrifugation over a Lymphoprep gradient (Gibco, Paisley, UK). Blood samples of 6 ml were diluted 1:2 with PBS and layered on an equal volume of Lymphoprep in a centrifuge tube. After centrifugation at 1000*g* for 20 min, gradient-separated lymphocytes were recovered, diluted 1:4 with PBS and centrifuged again at 1000*g* for 10 min at 4°C. The resulting cell pellets were resuspended in PBS and counted in a Neubauer chamber. Membrane integrity was assessed by the Trypan blue exclusion method. Aliquots of about 6×10^6 cells/ml were resuspended in freezing medium (90% FCS and 10% DMSO) and stored at −80°C. Four subjects were randomly chosen and an aliquot of their lymphocytes used for irradiation experiments. One hour before irradiation, lymphocytes were thawed at 37°C, washed twice, resuspended in cold RPMI medium and counted in a Neubauer chamber.

Irradiation of the cells

Irradiation was carried out under two different conditions. In the first experiment γ-rays from a ^{60}Co source (1.17 and 1.33 MeV) were used. The source-surface distance was 80 cm and the sample was placed at a depth of 5 cm in an ice-water bath to prevent undesired DNA repair; the dose rate was 0.2 Gy/min. In the second experiment high energy X-rays from a linear accelerator (Elekta SL75) with a maximum energy of 6 MeV were employed. The sample was placed at the linear accelerator isocenter (100 cm) at a depth of 5 cm in a ice-water bath, and the dose rate was 2.0 Gy/min. In both cases the absorbed dose determination was performed with a Farmer ionisation chamber, following the method described in the IAEA Report 398 [15]. This experiment was repeated twice

for lymphocytes from two different donors. With the third experiment performed using filtered low-energy X-rays. Irradiation was performed with a Roentgen-therapy unit operating at 200 kV and 20 mA (0.5 mm Cu filtration), dose-rate 1.1 Gy/min as measured with a Farmer ionisation chamber. The source-surface distance was 40 cm and the sample was placed at a depth of 2 cm in an ice-water bath.

Each cell type was irradiated with doses in a range from 1 to 10 Gy. All experiments were performed including a sample of non-irradiated cells (0 Gy), to obtain the background levels in undamaged cells, for each condition of irradiation.

Lymphocytes were irradiated in suspension in the first experiment. In the second and third experiment, lymphocytes and F10-M3 were irradiated after being embedded in agarose gel on microscopic slides for the comet assay.

For lymphocyte irradiation in suspension, the thawed cells were resuspended at a concentration of 5×10^6/ml in RPMI medium and aliquots of 200 µl were dispensed into micro centrifuge tubes and kept on ice during irradiation.

For the irradiation of the cells embedded in agarose, aliquots of the lymphocyte suspension or trypsinised tumour cells, containing about 100,000 cells, were centrifuged at 250*g* for 10 min and the resulting pellets were resuspended in LMA and layered on microscopic slides. During irradiation, the slides were kept in an open tank filled with pre-oxygenated RPMI medium. The tank was kept in an ice-water bath. Immediately after irradiation, the slides were placed in lysis solution and run through the comet assay.

Comet assay

The cells were resuspended, at the required concentration, in 1% LMA in PBS and transferred in a drop onto an agarose pre-coated slide, covered with a coverslip and allowed to solidify at 4°C. Two gels were accommodated on one slide and each experimental point was run in duplicate.

For the measurement of DNA breaks, the cells were then subjected to a lysis step (1 h incubation at 4°C in 1% *N*-lauroyl-sarcosine, 2.5 M NaCl, 100 mM Na_2EDTA, 1% TritonX-100, 10% dimethylsulfoxide, pH 10). After lysis, the slides were placed in an ice-cold electrophoresis chamber containing an alkaline electrophoresis solution (300 mM NaOH, 1 mM Na_2EDTA, pH 13) for 20 min to allow DNA unwinding. The electrophoresis was subsequently conducted for 20 min at 0.8 V/cm and 300 mA, at 4°C. At the end of the electrophoresis the slides were neutralised with 40 mM Tris–HCl, pH 7.4, stained with ethidium bromide overnight and analysed the following day. To prevent additional DNA damage, all the steps described above were conducted under dim light or in the dark.

To measure DNA oxidative damage, after the lysis step the slides were incubated at 37°C with the *Escherichia coli* enzyme FPG for purine oxidation detection (crude extract kindly provided by Professor Collins, University of Oslo, Norway). Two gels of the same sample were incubated with the enzyme (1:1000 dilution of the extract) for measurement of FPG-sensitive sites, and also with buffer only, for measurement of DNA SBs. At the end of the enzyme incubation (1 h), all slides were subjected to electrophoresis as described above.

Microscopic analysis was carried out by means of a Labophot-2 microscope (Nikon, Tokyo, Japan) provided with epifluorescence and equipped with a rhodamine filter (excitation wavelength 546 nm, barrier 580 nm). The images of 50 randomly chosen nuclei per slide were captured and analysed using custom-made imaging software coupled with a CCD camera (model C5985, Hamamatsu, Sunayama-Cho, Japan). For each image, the program calculated the total fluorescence (i.e. the sum of the grey levels of all pixels) distribution along the longer axis of the nucleus and the fluorescence distribution of the head and tail of the comet, respectively. Data were expressed as percent of total fluorescence migrated in the tail for each nucleus (percent of DNA in tail), which is linearly related to the number of DNA breaks. The mean percent of DNA in the tail of 50 nuclei per gel was calculated, and the duplicate values were further averaged. The value of percent of DNA in the tail obtained without FPG incubation estimated the basal number of DNA SBs, whereas specific oxidative damage on purines (FPG-sensitive sites) was assessed by subtracting the value found spin buffer-incubated slides from that obtained with FPG.

Statistical methods

The results were expressed as means ± SE. The GraphPAD software for Windows was used for statistical analysis (Prism 3.0). Correlations were performed using linear regression analysis.

Results

Figure 1 shows the dose-dependent relationship between γ-irradiation at low dose rate (0.2 Gy/min) and DNA damage, measured with the comet assay in lymphocytes irradiated in suspension. The number of DNA breaks expressed as percent of DNA in the tail was linearly related to the γ-ray dose.

The results obtained after irradiation with high energy X-rays from a linear accelerator at higher dose rate (2.0 Gy/min) of agarose-embedded human lymphocytes and F10-M3 cells, are shown in Figures 2 and 3, respectively. In both cases, the

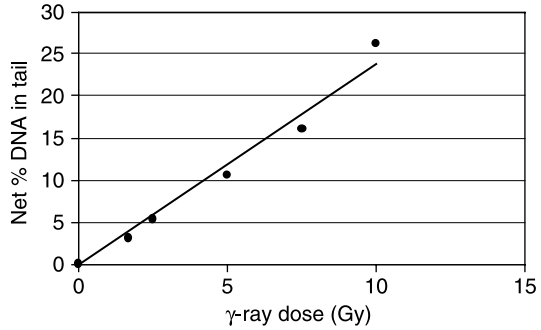

Figure 1. γ-Ray dose-response curve for lymphocytes. The cells were irradiated in suspension, before being run through the comet assay for the analysis of DNA damage. The values are expressed as net percent of DNA in the tail (i.e. the basal level corresponding to dose = 0 was subtracted from each value) and are plotted versus γ-ray dose. The equation of the linear regression analysis $y = 2.39 \times (R = 0.99)$.

number of DNA breaks induced by X-rays was linearly related to the radiation dose, and the regression lines obtained for lymphocytes and F10-M3 cells were not significantly different ($p = 0.51$ for slopes and $p = 0.74$ for intercepts).

A dose response curve identical to that obtained with X-rays from a linear accelerator was also obtained with copper-filtered low energy X-rays from a Roentgen-therapy unit. The corresponding equations were $y = 2.79 \times (R = 0.99)$ for the former and $y = 2.80 \times (R = 0.99)$ for the latter.

A comparison of the radiation dose-effect regression lines obtained for lymphocytes with γ- and X-rays from both sources showed that the slopes were not different ($p = 0.82$ for slopes and $p = 0.40$ for intercepts), indicating that under our experimental conditions the same dose of γ-, high energy X-rays and

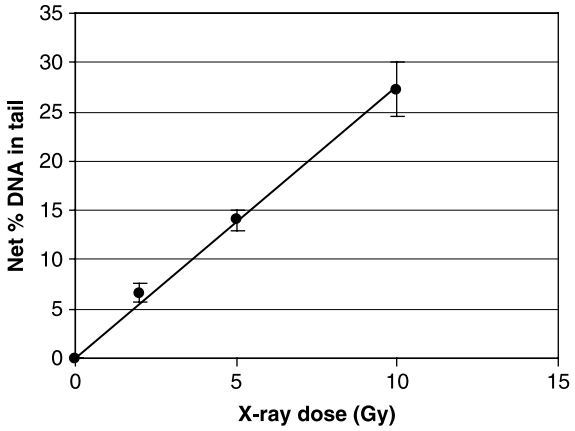

Figure 2. X-ray dose-response curves for lymphocytes. The cells were irradiated while embedded in agarose gel on microscopic slides, and run through the comet assay for analysis of DNA damage. The values are expressed as net percent of DNA in the tail (mean ± SE of two experiments) and are plotted versus the X-ray dose. The corresponding equation of the linear regressions analysis $y = 2.79 \times (R = 0.99)$.

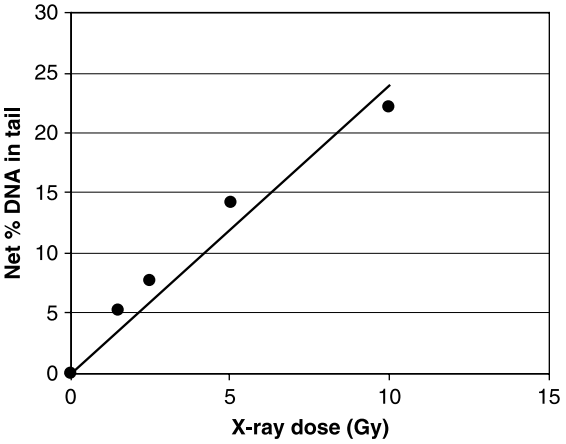

Figure 3. X-ray dose-response curves for F10-M3 cells. The cells were irradiated while embedded in agarose gel on microscopic slides, and run through the comet assay for analysis of DNA damage. The values are expressed as net percent of DNA in the tail (mean ± SE) and are plotted versus the X-ray dose. The corresponding equation of the linear regressions analysis is $y = 2.39 \times (R^2 = 0.97)$.

filtered low energy X-rays induced the same amount of DNA damage.

Based on these observations, a calibration curve was constructed, with the data obtained in irradiated lymphocytes, substituting the radiation dose with breaks frequency, based on the assumption that 1 Gy induces 0.31 breaks/10^9 Da DNA [12]. The corresponding equation ($y = 8.89 \times (R = 0.99)$, with y = %DNA in tail and x = DNA breaks per 10^9 Da DNA) allowed the conversion of the raw comet assay data into frequency of DNA breaks.

To verify that the employed dilution of FPG enzyme was enough for the enzyme activity to be saturated, but not giving rise to non-specific cleavage activity, the dependence of the number of enzymatically-induced breaks on the enzyme dilution was analysed in NCI-H520 cells (Figure 4). The results of these

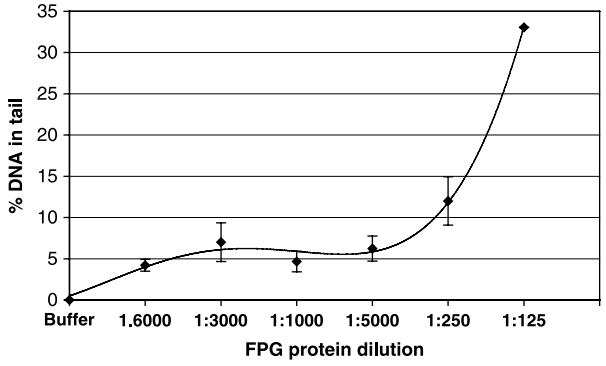

Figure 4. FPG activity at different enzyme dilutions. NCI-H520 cells in basal conditions were exposed to graded dilutions of the *E. Coli* extract containing the enzyme, and the induced breaks were measured with the comet assay. FPG-induced DNA breaks (FPG sites) are expressed as percent of DNA in tail (mean ± SE of eight experiments).

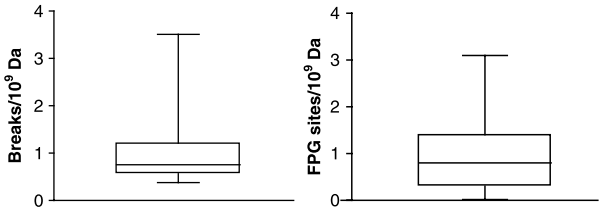

Figure 5. Calculated basal levels of DNA damage in human lymphocytes. Box and whiskers plot of the distribution of DNA breaks/10^9 Da DNA (left panel) and FPG sites/10^9 Da DNA (right panel) in 70 healthy volunteers, calculated by means of X-ray-calibrated comet assay (calibration curve in Figure 5). The upper and lower boundaries of the box are the upper and lower quartiles (1.21 and 0.57 for DNA breaks and 1.41 and 0.31 for FPG sites). The box contains the middle 50% of values. The horizontal line inside the box indicates the median: 0.76 for breaks/10^9 Da DNA and 0.80 for FPG sites/10^9 Da DNA. The vertical lines extend to highest and lowest levels that are not outliers: 3.51-0.38 for DNA breaks and 3.10–0.02 for FPG sites.

experiments showed that the enzyme was saturated between 1:3000 and 1:500 extract dilution, at lower dilutions the number of induced breaks increased again, likely due to non-specific cleavage activity.

Finally, the basal levels of DNA damage in human lymphocytes were calculated both for DNA breaks and FPG-sensitive sites using the obtained calibration curve. The distribution of the calculated levels of DNA breaks and FPG-sensitive sites in the group of volunteers that participated in this study are shown in Figure 5. For SBs, the mean ± SE was 0.9 ± 0.07 breaks/10^9 Da (corresponding to 0.6 ± 0.07 breaks/10^6 bp), and the median 0.76 breaks/10^9 Da DNA (geometric mean = 0.85, range 0.38–3.51, 25th percentile = 0.57, 75th percentile = 1.21). For DNA oxidation (FPG-sensitive sites), the mean ± spSE was 0.91 ± 0.08 FPG sites/10^9 Da DNA (0.6 ± 0.07 FPG sites/10^6 bp), and the median 0.80 FPG sites/10^9 Da DNA (geometric mean = 0.59, range 0.02–3.10, 25th percentile = 0.31, 75th percentile = 1.41).

DNA oxidation can also be expressed as FPG-sensitive sites per 10^6 guanines, using a previously reported calculation, based on the assumption that G represents 20% of the total DNA bases [16]. The average level of DNA oxidation in the analysed human lymphocyte sample was thus calculated to be 1.48 ± 0.13 FPG-sensitive sites per 10^6 guanines (mean ± SE), and the median as 1.30 FPG-sensitive sites per 10^6 guanines.

Discussion

Measuring DNA damage in living cells without artefacts is a critical issue, especially when studying basal levels of oxidation, which are in most cases relatively low.

Recently, an interlaboratory study (ESCODD, European Standards Committee on Oxidative DNA Damage) was set up to evaluate different approaches for detecting oxidised bases in DNA. The results showed that the background levels of base oxidation in human DNA (HeLa cells), measured with HPLC spand enzymic methods, were different by ~7-fold, although the discrepancy was lower than previously reported [5].

One crucial step when comparing data obtained with enzymatic methods with those obtained with HPLC is calibration. In the present study we used the calibration procedure and calculations proposed by Collins et al. [13], which was used in the ESCODD interlaboratory comparison. This procedure is based on the original work of Ahnstrom and Erixon [12], which employed γ-rays from ^{60}Co. We selected the same type of radiation in the present study, along with high energy X-ray radiation from a linear accelerator, which has biological efficacy similar to ^{60}Co radiation. As expected, the results obtained with these two different radiation sources were similar. Although different dose rates were used in the two experiments, they did not seem to affect the results, as confirmed by the alkaline unwinding measurements.

No difference was found in the response to irradiation of human lymphocytes and murine melanoma F10-M3 cells, indicating that under our experimental conditions the response is not dependent on cell type. Also, the results did not depend on the way cells were irradiated, i.e. attached to a dish or directly on comet assay agarose-coated slides. The homogeneity of the responses is probably due to the fact that the cells were maintained on ice throughout irradiation and post-irradiation, ruling out the possibility of DNA repair during irradiation: under these conditions different cells respond equally to the same radiation dose, regardless of the dose rate. Also, the brief period that the cells are in warm agarose does not seem to allow significant repair.

The calibration curve obtained in the present work differs from that published in a previous study, which reported substantially higher (about 2.5-fold) levels of DNA damage for the same interval of radiation doses [13]. This discrepancy might be partly explained by the type of radiation used by these authors, i.e. 200 kV unfiltered X-rays from a therapeutic X-ray machine. Similar results were obtained by Moller et al. [3] using a similar source of X-rays. The biological effectiveness of X-rays varies considerably with energy, and 200–250 kV X-rays have been shown to have a biological effectiveness relative (RBE) to ^{60}Co, ranging from 1.5 to 4, depending on the presence and type of filters and on the biological endpoint studied (chromosomal aberrations, cell transformation etc.) [17]. Adding filters to an X-ray beam selectively removes the low-energy, low-penetrating photons, with higher linear energy transfer (LET), i.e. more damaging per dose

unit. The importance of the presence of filters is further confirmed by our data showing that copper (0.5 mm) filtered 200 kV X-rays provide a calibration curve identical to that obtained with a linear accelerator.

Another difference between the present work and the previous studies is the duration of the unwinding and electrophoresis steps (20 + 20 min, respectively, in our case, 40 + 30 min in the other). We found that this increase in unwinding and electrophoresis time is accompanied by an increase (about 25%) in percent of DNA in tail (data not shown). Thus, this might also concur to the discrepancy among the two final calibration curves.

Using the calibration curve obtained in the present study, the background level (median value) of lymphocyte DNA damage in a group of healthy volunteers was estimated to be 0.76 breaks/10^9 Da DNA (0.50 breaks/10^6 bp) and 0.8 FPG sites/10^9 Da DNA (0.52 FPG sites/10^6 bp), corresponding to 1.30 FPG-sensitive sites per 10^6 guanines. These levels of human lymphocyte oxidative damage are about 4-fold higher than those reported in the ESCODD study for the comet assay and are closer to the reported levels of 8-oxo-7,8-dihydroguanine as measured by HPLC (4.24 per 10^6 guanines) [18].

In conclusion, on the basis of the described calibration for the comet assay, the basal levels of oxidative DNA damage in human lymphocytes from healthy volunteers resulted to be in the same order of magnitude as those measured with HPLC, i.e. 10^{-6} guanines. In view of the above mentioned sources of variability, ideally each laboratory should develop its own calibration curve, and the radiation employed should display the same biological efficacy than ^{60}Co. Whenever a curve made by others has to be used, it should be chosen on the basis of the most extensive similarity of the conditions of the assay.

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