J. Toxicol. Pub. Health
Vol. 20, No. 2, pp. 89-100 (2004)

Official Journal of
Korean Society of Toxicology
Available online at http://www.toxmut.or.kr

Exploitation of the Dose/Time-Response Relationship for a New Measure of DNA Repair in the Single-Cell Gel Electrophoresis (Comet) Assay

Byung-Soo Kim[1], Lutz Edler[2], Jin Joo Park[3], Dietrich von Fournier[4], Wulf Haase[5], Marie-Luise Sautter-Bihl[6], Egbert Hagmüller[7], Florian Gotzes[3] and Heinz Walter Thielmann[3]

[1]Department of Applied Statistics, Yonsei University, Seoul 120-749, Korea
[2]Biostatistics Unit R0700, German Cancer Research Center, Heidelberg, D-69120
[3]Division of Interaction of Carcinogens with Biological Macromolecules, German Cancer Research Center, Heidelberg, D-69120
[4]Gynecological Radiology, Radiological Clinic, University of Heidelberg, D-69115
[5]Clinic of Radiotherapy and Radiological Oncology, St. Vincentius Clinics, Karlsruhe, D-76132
[6]Clinic of Radiotherapy, Municipal Clinics gGmbH Karlsruhe, Karlsruhe, D-76133
[7]Surgical Department of the Heilbronn Clinics, Heilbronn, D-74078, Germany

Received February 6, 2004; Accepted April 6, 2004

ABSTRACT. The comet assay (also called the single-cell gel electrophoresis assay) has been widely used for detecting DNA damage and repair in individual cells. Since the conventional methods of evaluating comet assay data using frequency statistics are unsatisfactory we developed a new quantitative measure of DNA damage/repair that is based on all information residing in the dose/time-response curves of a comet experiment. Blood samples were taken from 25 breast cancer patients before undergoing radiotherapy. The comet assay was performed under alkaline conditions using isolated lymphocytes. Tail DNA, tail length, tail moment and tail inertia of the comet were measured for each patient at four doses of γ-rays (0, 2, 4 and 8 Gy) and at four time points after irradiation (0, 10, 20 and 30 min) using 100 cells each. The resulting three-dimensional dose-time response surface was modeled by multiple regression, and the second derivative, termed 2D, on dose and time was determined. A software module was programmed in SAS/AF to compute 2D values. We applied the new method successfully to data obtained from cancer patients to be assessed for their radiation sensitivity. We computed the 2D values for the four damage measures, i.e., tail moment, tail length, tail DNA and tail inertia, and examined the pairwise correlation coefficients of 2D both on the log scale and the unlogged scale. 2D values based on tail moment and tail DNA showed a high correlation and, therefore, these two damage measures can be used interchangeably as far as DNA repair is concerned. 2D values based on tail inertia have a correlation profile different from the other 2D values which may reflect different facets of DNA damage/repair. Using the dose-time response surface, other statistical models, e.g., the proportional hazards model, become applicable for data analysis. The 2D approach can be applied to all DNA repair measures, i.e., tail moment, tail length, tail DNA and tail inertia, and appears to be superior to conventional evaluation methods as it integrates all data of the dose/time-response curves of a comet assay.

Keywords: Quantitation of comet assay data, Dose-time-response surface, Second derivative, γ-radiation, Radiation sensitivity of cancer patients, Human lymphocytes.

INTRODUCTION

The single cell gel electrophoresis (SCGE) assay, also called comet assay, is a reliable and sensitive method of detecting DNA damage/repair induced by various genotoxic agents in individual cells. Only a few years ago has the comet assay been introduced for genotoxicity testing, and, within a short time, has become a standard technique for detecting DNA damage/repair. Today, the assay is being widely used in genetic toxicology (Tice *et al.*, 2000; Rajaguru *et al.*, 2001), environmental biomonitoring (Plappert *et al.*,

Correspondence to: Lutz Edler, Biostatistics Unit R0700, German Cancer Research Center, Im Neuenheimer Feld 280, D-69120 Heidelberg, Germany
E-mail: edler@dkfz.de

1997; Hartmann *et al.*, 1998; Mitchelmore *et al.*, 1998; Gichner *et al.*, 2000), occupational studies (Carere *et al.*, 2002; Geraj-Vrhovac *et al.*, 2000, 2002), clinical investigations (McCurdy *et al.*, 1997; Sarkariâ *et al.*, 1998; Morris *et al.*, 1999; Blasiak *et al.*, 2000; Zhang *et al.*, 2000), molecular epidemiology (de Restrepo *et al.*, 2000; Maluf *et al.*, 2001), human epidemiology (Berwick *et al.*, 2000) and nutrition (Gonzales *et al.*, 2002), and even forensic medicine (Johnson *et al.*, 2002). For a comprehensive review of the comet assay the reader is referred to Fairbairn *et al.* (1995).

Tice (1995) and Tice *et al.* (2000) discussed applications of the comet assay for detecting DNA damage/repair in individual cells, and various aspects of possible guidelines for the general use of comet assay in genetic toxicology. There are, however, several issues in statistical considerations that have to be resolved before the comet assay can be accepted as a standard assay for detecting DNA damage/repair in single cells. These are (i) to clarify how various forms of DNA damage can be quantified appropriately and what the limitations of the quantification are, (ii) to develop a statistical procedure for the dose-response relation from which criteria of an unequivocal response can be derived, and (iii) to validate the comet assay in order to distinguish genotoxic from non-genotoxic substances and, possibly, carcinogens from non-carcinogens. The purpose of this work is to present and discuss a new damage/repair measure which exploits the dose-time-response surface of the DNA repair kinetics and to evaluate its properties with respect to the appropriateness of quantification of DNA damage/repair. Our aim is to develop a comprehensive measure which is represented in the dose-time-response surface. We believe that defining the DNA damage/repair activity in terms of a two-dimensional slope is reasonable and is compatible with the concept of establishing an assay potency measure.

Since the introduction of the alkaline version of the comet assay the breadth of applications and the number of investigations using this technique have increased almost exponentially, and yet, comet assay data in most applications are presently not evaluated in an integrative manner, as most researchers only compare damage measures, e. g. tail moments or tail lengths, for one specific time point and one specific treatment level with control or standard samples.

The conventional statistical approach of evaluating comet assay data on DNA repair activity is to display sets of histograms ordered according to time after treatment (Plappert *et al.*, 1997), or to split a three-dimensional dose-time-response surface, if this is available at all, into several time course curves in two-dimensional panels for the doses applied (Gichner *et al.*, 2000), or to use the relative decrease of the damage measure (e.g., tail moment) instead of using the slope at which the damage measure decreased with time (McCurdy *et al.*, 1997). In contrast to the conventional approach we propose to use the second derivative of the dose-time-response surface, designated 2D, which is the slope of slopes, as a representative measure of DNA repair activity in cells. This single measure of the DNA repair activity combines all the information of the experiment, and delineates a way for comparing a whole set of dose-response data obtained from one individual with the corresponding whole set of another individual. Dealing with the usual wealth of comet assay data, this comprehensive term, 2D, is expected to be superior to a conventional point-wise comparison which focuses at distinct time points or dose levels.

We propose using a comprehensive measure of DNA repair which is represented in the dose-time-response surface. Defining the DNA repair activity in terms of a slope value determined on this surface is compatible with the concept of assay potency as it was promoted by the work of Margolin *et al.* (1988) for the assessment of mutagenicity. By combining two separate regression steps, of time and of dose, into one, we devise an efficient estimator. For calculation of the second derivative we have developed a user-friendly software program based on SAS/AF, “Comet Assay” which is run at Windows 95/98 platform with the SAS system (SAS, 1993). This program is available in the internet for free download from the ftp site, ftp://ftp.yonsei.ac.kr/pub/cometassay.

MATERIALS AND METHODS

Blood Samples

Citrated whole blood was collected from 25 breast cancer patients who had not received chemotherapy but were scheduled for radiotherapy. Blood samples were taken before the patients underwent radiotherapy. Patients were under clinical observation and treatment at the Clinics for Radiotherapy of the University of Heidelberg, of the St. Vincentius Clinics of Karlsruhe, and the Municipal Clinics of Karlsruhe, Germany. Preparation of lymphocytes has been described in Kim et al. (2002a). Informed consent had been obtained from each patient. This project was approved by the Ethics Commission of the Ruprecht-Karls-University Heidelberg (decision no. 37/98).

Preparation of Lymphocytes

Twenty ml of citrated blood were mixed with an equal

volume of balanced saline solution (constituents: 0.9% glucose, 45 μM $CaCl_2$, 0.9 mM $MgCl_2$, 14 mM NaCl, 5 mM KCl, 130 mM Tris-HCl, pH 7.6). Twenty ml of a ready-made solution of Ficoll-Paque (5.7 g Ficoll 400; 9 g sodium diatrizoate in 100 ml; Pharmacia Biotech, Freiburg, Germany) were placed in a 50-ml centrifuge tube and overlayered with 20 ml of the cell suspension mentioned above. Samples were centrifuged for 30 min at 600 x*g* and 20°C. After centrifugation, the uppermost layer, which contained plasma, was removed with a Pasteur pipette and discarded. The second layer (from the top), called buffy coat, containing lymphocytes, was carefully collected (4~5 ml) with a Pasteur pipette, transferred to a 50-ml tube, diluted with 45 ml of balanced saline solution, and centrifuged (350 x*g*, 20°C, 20 min).

Cell Treatment and Single-cell Gel Electrophoresis Assay (Comet Assay)

The comet assay was performed under alkaline conditions essentially following the procedure described by Singh *et al.* (1988). Immediately after irradiation, cells were subjected to the comet assay. To allow DNA repair to go on, tubes were incubated at 37°C for 10, 20 and 30 min (control: 0 min). Frosted microscope slides (76 mm x 26 mm) were precoated with 85 μl of 0.8% low melting agarose (Merck, Darmstadt, Germany) using a 24-mm x 50-mm coverslip, and kept for 1 h on an ice-cold and wetted metal supporter to facilitate solidification of the agarose. After removing the coverslip, cells from individual tubes were mixed with 85 μl of 0.7% low melting agarose at 37°C, and the suspension was spread on the precoating layer. The slide was cooled for 3 min on ice with the coverslip left on the surface. Then, coverslips were removed and the slides were immersed in precooled (4°C) lysing solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-HCl at pH 10, 1% Triton X-100 and 10% dimethyl sulfoxide; the latter two were always added freshly) and kept for 1 h. After lysis, slides were drained and placed in a horizontal gel electrophoresis chamber (model GE-A3-1, Angewandte Gentechnologie-Systeme GmbH, Heidelberg, Germany) filled with electrophoresis solution (300 mM NaOH, 1 mM sodium-EDTA at pH 13). In this solution, DNA unwinding and breakage of alkali-labile sites took place for 20 min before the power supply was turned on. Electrophoresis proceeded for 25 min at 25 V, the current was adjusted to 300 mA by increasing or decreasing the level of the electrophoresis solution. After electrophoresis, slides were immersed for 5 min in neu*tralization buffer* (0.4 M Tris-HCl at pH 7.5), stained with 45 μl ethidium bromide (20 μg/ml), covered with a coverslip and stored in a sealed box at 4°C until analysis. Each slide was analyzed by randomly choosing 50 cells (two slides were available for each experimental point) using the analysis system described below.

Analysis System

The objects were observed with a Leica Dialux 20 fluorescence microscope (magnifications: objective, 40×; eyepiece, 10×; camera factor, 0.63) which was equipped with a Leica type 307-148.002 fluorescence attachment, an excitation filter of 515~560 nm, a barrier filter of 590 nm, and a 100-W mercury lamp (Leica, Bensheim, Germany). The microscope was attached to a COHU high-performance CCD camera (model 4912-5000; COHU, San Diego, CA, USA) connected to a Leica Q500IW computer. The analysis software, Komet 4.0, was from Kinetic Imaging (Liverpool, UK).

Measuring the Comet

The comet assay uses a micro-electrophoretic technique for the direct visualization of DNA damage in individual cells. A small number of irradiated cells suspended in a thin agarose gel on a microscopic slide is lysed, electrophoresed at neutral pH, and stained with a fluorescent DNA binding dye. Upon heating or treatment with alkali, the double stranded DNA will unwind and separate into single strands. As the agarose gel solidifies, a gel matrix forms that consists of long tangled chains of polymers and there are interconnecting channels or pores between the matrix of the gel. During electrophoresis negatively charged DNA fragments move toward the anode at a rate that is inversely proportional to their length. Thereby, the electric field pulls the DNA fragments from the nucleus toward the anode. Longer DNA fragments will stay close to the nucleus, smaller fragments will move farther, and eventually DNA molecules are separated according to their size. When stained with ethidium bromide the DNA shows the shape of a comet. Migration parameters such as distance from the nucleus are used to determine the extent of DNA damage. Singh *et al.* (1988) and Olive *et al.* (1990) independently modified the method by developing alkaline versions of this assay using pH>13 and pH~12.3, respectively.

It has been well established that single- and double-strand breaks induced by ionizing radiation are efficiently repaired along the time course such that 50 percent of damage are repaired within 15 min and almost complete repair occurs within 1-2 h (Tice, 1995). It was also observed that after x-ray irradiation DNA repair was most actively performed within the first 15 min (Singh *et al.*, 1988). Therefore the time from the start of electrophoresis until the measurement of the comet is a

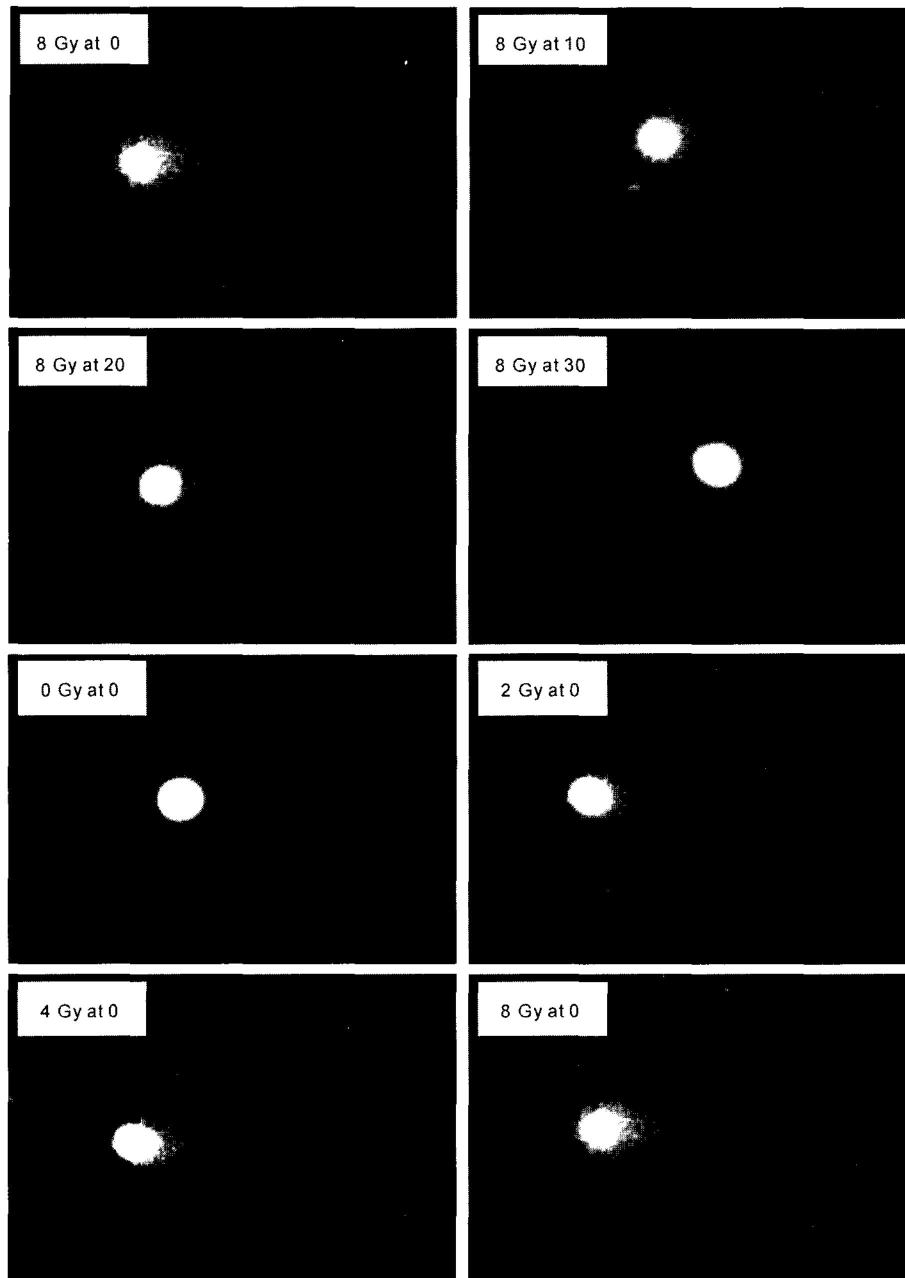


Fig. 1. Comet pictures of 8 Gy x-ray irradiation at times 0, 10, 20, 30 min and of 0, 2, 4 and 8 Gy at time t = 0.

crucial design parameter. Figure 1a-d exhibits real comets as a result of x-ray irradiation. Cells were treated with 8 Gy and incubated for 0, 10, 20 and 30 min, respectively.

One of the most important issues in the evaluation of the outcome of a comet assay is the definition of the most appropriate measure of the "comet". A valid measure should account for the fact that the extent of DNA liberated from the head of the comet is a function of the dose of the test agent (Fairbairn *et al.*, 1995; Tice, 1995). Figure 1e-h shows four comets obtained from cells treated with 0, 2, 4, and 8 Gy of x-ray irradiation, respectively, and incubated for 0 min. Cells irradiated with increasing doses display DNA comets of increasing length.

We briefly review the four most used measures that have been proposed for quantifying DNA damage: the tail DNA (Olive *et al.*, 1990), the tail length (Tice *et al.*, 1995; Ashby *et al.*, 1995), the tail moment (Olive *et al.*, 1990), and the tail inertia (Hellman *et al.*, 1995). The tail length can be defined in four different versions as demonstrated in Fig. 2:

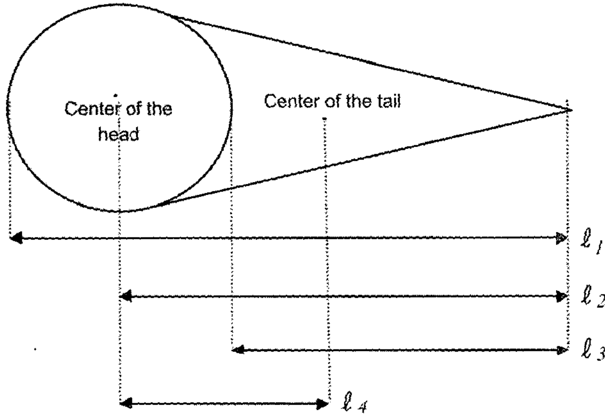


Fig. 2. Schematic figure of a comet and four versions of the tail length.

$$TL = \text{tail length (four versions } l_1\text{-}l_4 \text{ are defined graphically in Fig. 2)} \quad (1)$$

The tail DNA is calculated as percentage of DNA fragments in the tail value using areas and intensities of the tail and the head of the comet. The tail moment was originally defined as the product of the tail DNA and the tail length l_2. However, for defining the tail moment, Hellman *et al.* (1995) used the product of the tail DNA and the l_3 version of the tail length, calling it tail distance. We use this definition in our work. For defining the tail inertia, one first dissects the tail area into a certain number of local areas A_i, and for each local area one then calculates the local center. After that, the distances D_i between the head center and the various local centers are determined. Formulae for tail DNA, tail moment, and tail inertia are given in equations (2) to (4) below:

$$TD = \text{tail DNA} = \frac{TA \cdot TAI \cdot 100}{TA \cdot TAI + HA \cdot HAI}, \quad (2)$$

$$TM = \text{tail moment} = \text{tail DNA} \cdot l_3, \quad (3)$$

$$TI = \text{tail inertia} = \frac{\sum_i A_i \cdot AI_i \cdot D_i^2 \cdot 100}{TA \cdot TAI + HA \cdot HAI}, \quad (4)$$

where

TA = tail area,

TAI = tail area intensity,

HA = head area,

HAI = head area intensity,

A_i = area of the i-th local area,

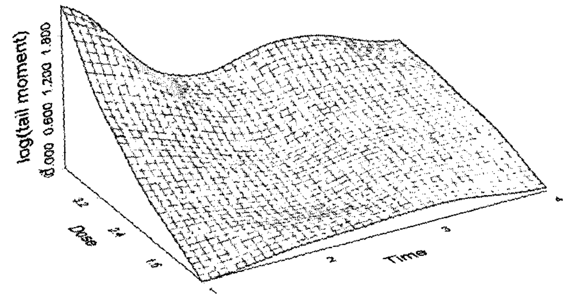
AI_i = average intensity of the i-th local area,

D_i = distance between the head center and the i-th local center.

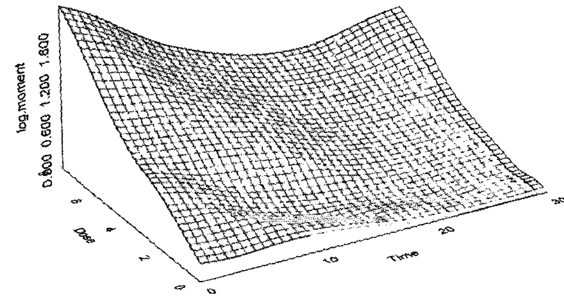
Hellman *et al.* (1995) discuss various aspects of these four measures, in general, and tail moment and tail inertia, in particular.

Experimental Design and Data

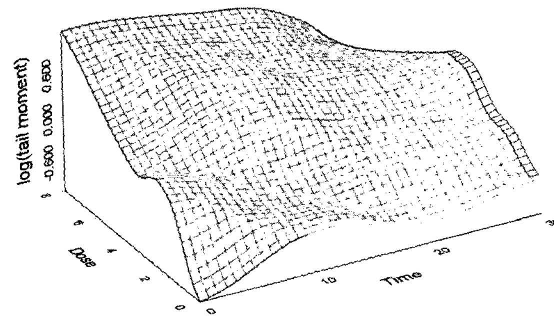
A single comet assay in our experiment consists of assay data obtained using lymphocytes from each individual. The DNA damage was measured at four doses of γ-rays (0, 2, 4, and 8 Gy), and for each dose, four time intervals after irradiation were used (0, 10, 20, and 30 min). One hundred cells were scored for each of the 16 dose-time combinations. Thus, by design, an experiment using lymphocytes from an individual defines a dose-time-response surface which results from the



3-D plot of log (tail moment) for patient 5



3-D plot of log (tail moment) for patient 4



3-D plot of log (tail moment) for patient 20

Fig. 3. Three dose-time-response surfaces of log(tail moment) for three patients: Patient no. 5 (a), patient no. 4 (b), patient no. 20 (c).

1,600 observations. A representative dose-time-response surface is shown in Fig. 3 when an appropriate smoothing algorithm is applied on the 4×4 grid of the 16 dose-time points. We employed the four DNA damage measures mentioned above: tail moment (TM; Olive *et al.*, 1990), tail DNA (TD; Olive *et al.*, 1990), tail length (TL; Tice *et al.*, 1995; Ashby *et al.*, 1995), and tail inertia (TI; Hellman *et al.*, 1995). For the tail length we followed the recommendation of Ashby *et al.* (1995) and used the distance l_3 between the trailing edge of the head and the end of the tail.

Data Transformation

Any statistical analysis that is free from biased estimates and is as accurate as possible in terms of standard errors, requires that quantitative measures are evaluated in their most suitable scale, e.g., the data should follow a Gaussian normal distribution when a statistical regression approach is chosen for data analysis. If the scale of the original data is not suitable for the planned analysis, data transformations are used to achieve a suitable scale as best as possible. In preliminary analyses we found that the various damage measures exhibited right skewness. There is a tendency of higher distance values lying far right of the median; lower values concentrated at a smaller distance left of the median. In addition, the distribution of values is naturally truncated at the left since negative values of the DNA damage/repair measures do not occur.

Logarithmic transformation of the original data have been used successfully in similar situations. However, we observed a non-negligible number of zero values (Table 1). Therefore, transforming the various damage measures into the log scale zeros is impossible since log(0) is an undefined number. We furthermore noted that the distribution of the number of zeroes for all twenty-five patients was not homogeneous with respect to damage measures, and, that the range of non-zero minima differed among damage measures. Given these facts we felt that it was reasonable to assign for "log(0)" the log(non-zero minimum)-1 whenever the measure value was zero. For example, when the non-zero minimum of the tail moment for a certain patient is equal to 0.01, we assign log(0.01)-1 = -5.61 for "log(0)".

We realized that the systematic parts, the dose and the time, could be removed from the response and the pure noise part, statistically expressed as residuals (observed value predicted value) could be checked for a normal distribution. By analyzing the residuals we observed for our damage measures and for the majority of the patients that the modified log transformation of the damage measure provided a closer fit to the normal distribution than the untransformed data. This observation is corroborated by the findings of Bauer *et al.* (1998) who reported that the (untransformed) tail moment followed the chi-square distribution. Taking a log transformation of the chi-square random variable one can get a distribution closer to the normal distribution.

Modeling Dose-time-response Surface: The Second Derivative

In a first step, one may calculate the slope of each damage measure within time separately for each dose. Due to continuous repair with time this slope is negative in general indicating a decrease of the damage measure with respect to time. In a second step one may compute the rate at which the slopes, calculated for each dose, decrease as dose increases. This rate is actually the second derivative of the dose-time-response surface (for an example see Fig. 3) with respect to the dose and time. Consequently, we propose the second derivative with respect to dose and time

$$2D = \frac{\partial^2 f(x, t)}{\partial x \partial t} \quad (5)$$

as the new measure of DNA damage/repair kinetics in the comet assay, where f(x, t) denotes the damage measurement of the comet assay at dose x and time t. This second derivative, 2D, reflects how fast the slope during recovery from DNA damage decreases as dose increases.

Simultaneous multiple linear regression on dose and time shapes 2D statistically. Before presenting the multiple regression model, below, we illustrate the 2D concept as a two step approach. Firstly, at each dose level, one may analyze the time course of DNA damage at four different time points and determine the slope in a simple linear regression, with time as an explanatory

Table 1. Distribution of the number of zero values out of 1600 observations and the range of nonzero minimum for all twenty five patients for the four DNA/damage repair measures tail moment (TM), tail length (TL), tail DNA (TD), and tail inertia (TI)

Damage measure	Minima	First quartile	Median	Third quartile	Maxima	Range of nonzero minima
TM	2	27	36	50	88	0.01-0.05
TL	8	42	59	85	119	1.00-2.00
TD	1	22	29	36	67	0.01-0.03
TI	0	0	0	1	25	39.59-1601.01

variable. When examining the tail moment TM as a generic variable on (modified) log scale, this regression model can be written as

$$\log(TM) = \beta_{0x} + \beta_{1x}t + \varepsilon_x, \quad (6)$$

where t denotes the time, β_{1x} the slope value of interest and β_{0x} the intercept, both depending on the fixed dose x at which this regression is evaluated. ε_x denotes the error term. In order to account for early damage/repair effects of the first 15 min, this analysis is modified by introducing a second explanatory variable, namely a time indicator, *time.indic* into the regression model. Therefore, we define

$$\text{time.indic} = \begin{cases} \text{time}, & \text{if time} > 15\ [\text{min}] \\ 0, & \text{if time} \leq 15\ [\text{min}], \end{cases} \quad (7)$$

and we generalize the model equation (6) to

$$\log(TM) = \beta_{0x} + \beta_{1x}t + \beta_{2x}\,\text{time.indic} + \varepsilon_x, \quad (8)$$

where the additional regression coefficient β_{2x} accounts for the time effect, which is a so-called nuisance effect in statistical terms. The regression coefficient β_{1x} is still the key parameter which represents the slope of the time course of DNA. Secondly, we model the slope parameter β_{1x} itself in a second regression model, with the dose x as the explanatory variable

$$\beta_{1x} = \gamma_0 + \gamma_1 x + \eta. \quad (9)$$

In this equation, γ_0 and γ_1 are again the regression coefficients and η is the normally distributed error term. An estimate of the regression coefficient γ_1 in equation (9) using the dose-response results obtained in the first set then yields automatically an estimate of the second derivative 2D.

Using multiple regression on dose and time simultaneously, the stepwise estimation procedure for 2D, described above, can be comprehensively performed in a multiple regression approach. This application uses the mathematical fact that the second derivative 2D calculated in the space of the dose-time points (x, t) is symmetric with respect to the order of differentiation under very general mathematical regularity conditions. Therefore, we suggest to determine 2D in the multiple regression model

$$\begin{aligned} Y_{ijk} &= \beta_{0i} + (\gamma_0 + \gamma_1 x_i + \eta_i)t_j + \beta_{2i}t_j^* + \varepsilon_{ik} \quad (10) \\ &= \beta_{0i} + \gamma_0 t_j + \gamma_1 x_i t_j + \beta_{2i}t_j^* + \varepsilon_{ijk}^*, \end{aligned}$$

where Y_{ijk} denote the response of the comet assay (any *damage measure or its transformation*) corresponding to the k-th comet of a cell at the i-th dose level and the j-th time point for i = 1, 2, 3, 4, j = 1, 2, 3, 4 and k = 1, ..., 100, with x_i denoting the i-th dose level and t_j the j-th time point. Define t_j^* as in (7): $t_j^* = t_j$, if $t_j > 15$ [min], and $t_j^* = 0$, if $t_j \leq 15$ [min]. ε_{ijk}^* is an aggregated error term which is the sum of $\eta_i t_j$ and ε_{ik}. The parameters β_{0i}, γ_0, γ_1, and β_{2i} denote again the regression parameters which are then estimated from the data set, i.e., from the 1600 individual cell data we had determined for each patient. The resulting estimate of the parameter γ_1 represents the second derivative with respect to dose and time. One can easily construct a design matrix in the form of $Y = X\beta + \varepsilon$ from equation (10) and apply the general linear model theory (Draper and Smith, 1998). For the computation we recommend the software module "Comet Assay" mentioned above a SAS/AF-based software program (SAS, 1993) specifically customized by us to compute the 2D measure.

RESULTS

The data that we are considering represent the comet assay outcomes in lymphocytes, measured at four different time points after irradiation with varying doses of γ-rays. A detailed description is given in the Materials and Methods section. As a routine we have 100 cells for each dose-time combination. Each experiment is defined to be a dose-time-response surface resulting from the 1600 observations. The dose-time-response surface can be presented graphically as shown for three selected experiments in Fig. 3.

We computed the 2D measure for the four damage measures for each of 25 patients using the "Comet Assay" program (Table 2) which includes the multiple regression as described above both for the unlogged and the modified log scale. As an example we explain here the results of the stepwise procedure (6)-(9) which we apply to the comet assay data obtained for the sample of patient 4 (see also Fig. 3). Table 2 gives the estimates of the parameters β_{0x}, β_{1x}, β_{2x} in the linear regression model (8) when the tail moment measures are evaluated on the modified log-scale. The estimates of β_{1x} are zero for the control x = 0 and negative for the three doses x = 2, 4, 8 with a tendency to increase with dose in absolute values. The values for β_{2x} represent reflect the adjustment for the early effect during the first 15 min. By applying the data in Table 3 to the regression model in equation (9) we obtain the estimated regression equation (11) as

$$\hat{\beta}_{1x} = -\,0.0038 - 0.0099x, \quad (11)$$

where $\hat{\beta}_{1x}$ represents the predicted value of the mean of β_{1x}. The regression coefficient $\gamma_1 = -0.0099$ in equation (11) is the estimated value of 2D for the tail

Table 2. 2D (second derivative) measures of tail moment (TM), tail length (TL), tail DNA (TD), and tail inertia (TI). 2D calculated for 25 patients in unlogged and (modified) logged scale

Patient	2D Measures							
	Unlogged				Logged[a]			
	$2D_{TM}$ [b]	$2D_{TL}$ [c]	$2D_{TD}$ [b]	$2D_{TI}$	$2D_{logTM}$	$2D_{logTL}$	$2D_{logTD}$	$2D_{logTI}$
1	-7.14	-4.39	-10.39	-11.85	-11.3	-2.4	-8.0	-4.3
2	-4.15	-1.74	-6.87	-13.46	-10.4	-5.0	-9.5	-4.2
3	-1.57	-0.68	-3.35	-9.30	-10.8	-5.3	-11.5	-2.7
4	-8.65	-2.31	-12.28	-11.99	-9.9	-1.1	-8.1	-4.6
5	-9.56	-5.27	-16.68	-16.62	-18.9	-9.3	-15.9	-6.7
6[d]	-14.73	-8.95	-17.63	-32.96	-18.2	-11.2	-11.4	-10.3
7	1.88	0.95	2.04	4.31	6.4	6.0	6.7	12.0
8	-20.42	-5.67	-21.19	-102.03	-14.8	-3.2	-6.9	-21.9
9	-2.21	-1.99	-3.65	4.87	-7.6	-8.4	-7.4	0.7
10	3.45	-0.22	2.63	0.16	-6.4	-6.3	-4.4	-2.5
11	-5.55	-3.54	-5.22	-19.48	-12.2	-8.2	-8.9	-5.2
12	-0.81	-2.46	-2.35	-0.35	-13.0	-10.1	-12.2	-1.2
13	1.27	-1.18	1.25	5.44	-12.1	-10.4	-11.2	-0.1
14	-1.99	-2.89	-2.59	-5.67	-12.7	-9.6	-11.5	-1.3
15	-6.27	-4.59	-8.34	-19.01	-20.0	-12.2	-16.5	-5.2
16	-2.70	-2.40	-2.79	0.38	-10.4	-5.7	-9.2	0.0
17	4.01	1.05	6.23	1.10	-2.8	0.4	-2.8	-0.7
18	-0.45	-2.04	-3.09	14.16	-10.1	-10.3	-10.6	2.2
19	4.71	1.68	3.49	14.35	4.1	-0.9	2.4	1.6
20	-1.05	-0.90	-2.22	-11.45	-4.4	-5.8	-2.2	-3.6
21	-6.85	-3.83	-6.34	-23.05	-15.4	-10.2	-12.9	-3.7
22	1.13	0.07	-0.59	8.86	-0.9	-1.8	-2.1	2.3
23	-5.12	-4.44	-7.10	-4.07	-18.0	-1.4	-15.9	-1.0
24	-6.39	-3.11	-8.42	-11.97	-15.1	-7.1	-14.1	-2.9
25	-0.21	-0.29	-0.29	2.40	-4.3	-2.7	-5.1	0.7

[a]Multiplied by 1000.
[b]Multiplied by 100.
[c]Multiplied by 10.
[d]Only 75 cells were evaluated for 4 Gy and 0 min.

Table 3. Estimated regression parameters β_{0x}, β_{1x}, and β_{2x} of the linear regression (8) for the modified log tail moment data of patient 4

Dose	β_{0x}	β_{1x}	β_{2x}
0	0.2275	0.0000	-0.0091
2	0.6842	-0.0427	0.0188
4	1.0064	-0.0218	-0.0047
8	2.2761	-0.0884	0.0388

Table 4. The correlation matrix of 2D measures for the modified logged (upper triangular and for the unlogged measures (lower triangular)

	$2D_{logTM}$	$2D_{logTL}$	$2D_{logTD}$	$2D_{logTI}$	
$2D_{TM}$		0.7020	0.9494	0.6249	$2D_{logTM}$
$2D_{TL}$	0.8739		0.7265	0.3342	$2D_{logTL}$
$2D_{TD}$	0.9703	0.8732		0.4199	$2D_{logTD}$
$2D_{TI}$	0.8652	0.6237	0.7665		$2D_{logTI}$
	$2D_{TM}$	$2D_{TL}$	$2D_{TD}$	$2D_{TI}$	

moment of patient 4. The value of 0.0099 is also found in Table 2 as $2D_{logTM}$ value in the row of the damage/repair measures calculated for patient 4.

Table 2 lists tail moment, tail length, tail DNA and tail inertia as four different damage/repair measures representing characteristic properties of the geometric shape of the comet. We addressed interdependency between these four measures in a correlation analysis and calculated the Pearson product correlation coefficients for all pairings of the four types of 2D values of Table 4, namely, $2D_{TM}$, $2D_{TL}$, $2D_{TD}$, $2D_{TI}$ and $2D_{logTM}$, $2D_{logTL}$, $2D_{logTD}$ and $2D_{logTI}$, respectively. The correlation matrix of the four measures is given in Table 4 both for the modified log scale and the unlogged scale.

This observation is supported by a principal component analysis (see Kim *et al.*, 2002b). Finally, the correlation between 2D measures were lower in the modified log scale than in the unlogged scale.

DISCUSSION

Based on the current state of knowledge, an expert panel recently reached consensus as to the most

appropriate methodology the be used when applying the comet assay to genotoxicity (DNA damage/repair) testing (Tice *et al.*, 2000). Regarding evaluation of results, these authors stated: "There was no consensus among the expert panel as to the most appropriate statistical method(s) to use other than an agreement that the analysis must be based on individual (cell) culture response." In other words, there is a need for developing original biostatistical evaluation methods for the comet assay. We want to emphasize that our proposed 2D value aims at evaluating the individual (cell) culture response in such a way as to gain a quantitative measure of DNA damage/repair.

The 2D measure that we propose here is superior to other descriptive evaluations in the sense that it integrates into a single number all the information contained in the dose-time response surface of an experiment. Numerous recent publications illustrate that evaluation of comet assays is done in a mere "yes" or "no" manner and DNA migration in the comet is described in purely qualitative terms, e.g., it is stated to be "significant" or, at best, "dose-related", without elaborating a quantitative term condensing the individual dose-response data to one measure. This is unsatisfactory because by this qualitative approach precious information is lost and neither DNA damaging potency of a substance nor cellular sensitivity can be gauged.

Would we have analyzed our data descriptively and accounted separately for the time since incubation and for the x-ray irradiation dose we would have obtained a panel of statistical results, displayed e.g., in sets of histograms as shown by the trellis plot in Fig. 4 for the patient 4 whose data served as an example throughout this paper. Obviously, a useful statistical evaluation must be based on meaningful data modeling. Based on all observations obtained for an individual a more statistically meaningful characterization of that individual's repair capacity is obtained with the second derivative

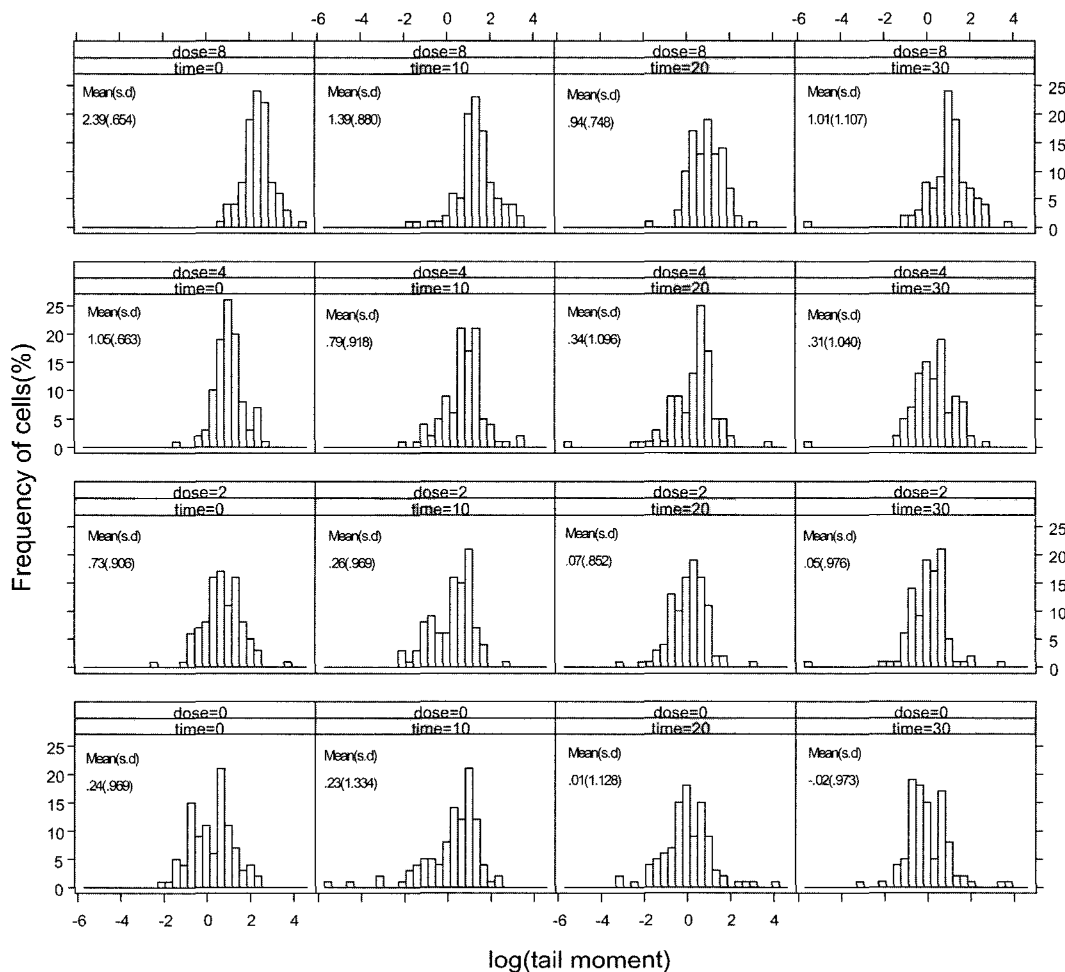


Fig. 5. Histogram of log(tail moment) at each dose-time combination for patient no. 4. Mean and standard deviation based on the 100 observations at each dose-time combination are shown at the upper left part of each panel.

2D. The damage/repair measure 2D is therefore in line with at least one of the demands formulated by Tice (1995): *He noted with respect to the appropriateness of* quantification of DNA damage that there were almost as many methods for quantifying DNA damage as there were scientists and he also wrote "...the most appropriate means of statistical analysis needs to be evaluated. The next few years should see these issues resolved."

The regression model on which the calculation of the 2D measure was based allows a comprehensive statistical evaluation of all available dose-time response data. Statistical methods based on selected subsets can hardly be as efficient in information extraction. McCurdy *et al.* (1997) simply considered the kinetics from 0 to 60 min of radiation-induced damage/repair for one dose level (1.5 Gy) and compared tail length measurements statistically only at one time point (30 min).

Plappert *et al.* (1997) used two exposures of 1 Gy each and presented the distribution pattern of their data as separate histogram plots for five time points (0, 30, 60, 90, and 120 min). Their measure of repair capacity defined as relative decrease (%) of the percentage of the damaged cells after 30, 60, 90, and 120 min can be directly related to our approach. It is, in a unit time scale, the ratio of the slope to the intercept β_{1x}/β_{0x}, following the notations in our equations (6) or (8), respectively. It remains to be investigated whether the slope measure β_{1x} or the ratio of the slope to the intercept β_{1x}/β_{0x} will be a better measure of the DNA repair activity. *Our multiple regression approach provides the suitable* framework and allows the extension to more than one exposure dose.

Sakaria *et al.* (1998) assessed the utility of both the comet assay and the pulsed field gel electrophoresis (PFGE) assay for the prediction of radiosensitivity in 23 non-transformed human fibroblast cell lines. Tail moment data of the comet assay for 50 cells were analyzed using the slope of the dose-response curve which was specified for 0, 30, 60, 90, 120, and 150 Gy at one time point (4 h). Surprisingly, the correlation between the comet assay slopes and the $D_{0.01}$ measure of the standard clonogenic survival dose-response assay was low and statistically insignificant. It would be interesting to see whether this failure of predicting radiosensitivity would have persisted if more than one time point or a dose-time-response surface measure had been used for the evaluation of the correlation.

The usefulness of the new proposed 2D measure ought to be intelligible for reasons of statistical modeling of the experimental data. Whether the second derivative of the dose-response surface of the comet assay outcome will show a high correlation with the clinical radiation sensitivity data will be a matter of practical application but not a test mark for the utility of the 2Ds. Finding out whether or not DNA repair as represented in the 2D values, correlates with clinical radiation sensitivity is the ultimate goal of our investigation. It is our hypothesis that there might be a correlation but there might as well be no correlation and yet the second derivative of the dose-response surface can be useful. Unfolding the various clinical aspects of radiation sensitivity and some of its possible causes, including multivariate analyses of the clinical data, would clearly exceed the framework of the present communication. This work must be left to further research. Nevertheless, we are deeming it mandatory first to condense the unwieldy wealth of comet assay data into one term to obtain a practical and accurate measure of DNA damage/repair. This is a useful undertaking of its own from which users of the comet assay will benefit. An example from a related field of application may illustrate this thought. Recently, Sasaki *et al.* (1999) addressed the aspect of the validation of the comet assay based on the 30 aromatic amines selected from IARC groups 1, 2A, 2B and 3 carcinogens and from the US National Toxicology Program database. These authors reported that their comet assay results showed a high positive response rate for rodent carcinogens and a high negative response rate for rodent genotoxic non-carcinogens.

The validity of a statistical estimation procedure *always depends on the validity of assumptions made on* the statistical distribution of the underlying data. The determination of the 2D measure using multiple linear regression assumed that the damage measure, i.e., tail moment, tail length, tail DNA or tail inertia, followed the normal distribution. The validity of these assumptions should be checked, for example with residual plots. We used this distribution check and found that the modified log-transformed data were more appropriate than the original data. When the response of the comet assay is represented by the frequency or the proportion of the damaged cells, as it is done sometimes (see e.g., Plappert *et al.*, 1997), one can still apply the regression model, but one has to consider other data distributions in a regression model. The generalized linear model (see, e.g., McCullogh and Nelder, 1989) provides the framework to extend our method for those response data.

Our analysis resulted in statistical estimates of the 2D damage repair measures (Table 3). Furthermore, we obtained the standard error of each estimated 2D and the p-value of its statistical significance from the "Comet Assay" software output. By using the p-value one may

test $H_0 : \delta = 0$ *versus* $H_a : \delta < 0$, where δ stands for the 2D value of the dose-time-response surface of an experiment (patient). A formal statistical test of the hypothesis $H_0 : \delta = 0$ is obtained using standard statistical methods. This approach can be extended easily to test for differences of DNA damage/repair capacity between groups of individuals, e.g., patients *versus* non-diseased controls. In our approach one has simply to add, in the multiple linear regression (10), a term denoting the adherence to one of the populations and test for its significance. The statistical theory of the general linear model provides the necessary methods. Obviously, covariates, for example the age of the persons investigated for DNA damage/repair can be included easily in this model.

ACKNOWLEDGEMENTS

The experimental work in this project was supported by the Bundesamt für Strahlenschutz, Projekt StSch 4116. B.-S Kims and L. Edlers works were jointly supported by Korea Science and Engineering Foundation (KOSEF, Project number 2000-104-01-2) and Deutsche Forschungsgemeinschaft (DFG) through KOSEF-DFG bilateral agreement. L. Edler's work was partially also supported by NATO Science Program Pilot Study 2 on Advanced Methods in Carcinogenic Risk Assessment.

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