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CONSTRUCTION AND VALIDATION OF A DOSE-RESPONSE CURVE USING THE COMET ASSAY TO DETERMINE HUMAN RADIOSENSITIVITY TO IONIZING RADIATION

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Individual radiosensitivity is an individual characteristic associated with an increased reaction to ionizing radiation. The purpose of our work is to establish a dose-response curve useful to classify individuals as radiosensitive or radioresistant. Thus, a dose-response curve was constructed by measuring in vitro responses to increasing doses (0 to 8 Gy) of gamma radiation in the comet assay. The obtained curve fit well with a linear equation in the range of 0 to 8 Gy. The overall dose-response curve was constructed for percent DNA in tail, as a measure of the genetic damage induced by irradiation. To probe the goodness of the constructed curve, a validation study was carried out with whole blood from two donors in a blind study. Results show that, for the two applied doses (2 and 6 Gy), the obtained values fit well inside the interval of confidence of the curve. In conclusion, our results demonstrate the usefulness of the comet assay in determining individual responses to defined doses of gamma radiation. The standard dose-response curve constructed may be used to detect individuals departing from reference values.

Individual radiosensitivity is a personal characteristic associated with an increased reaction to ionizing radiation (Twardella and Chang-Claude 2002). Exposure of humans to effective doses of ionizing radiation presumes a heterogeneous response affecting both acute and delayed effects as well as cancer. There are radiosensitive subgroups showing a greater incidence of these effects (Fernet and Hall 2004). Individual radiosensitivity characterization is important not only from the perspective of the radiological safety but also from the radiotherapy point of view. It needs to be pointed out that adverse effects characterizing radiosensitive individuals are mainly influenced by the genetic individual background (Wang et al. 2005).

Different in vitro approaches on how to measure individual radiosensitivity have been proposed. Thus, different methodologies such as the clonogenic assay**,** the frequency of chromosome aberrations (CA), chromosome painting techniques, the frequency of micronuclei (MN), and the capacity of DNA repair residual damage, among others, were proposed (Scott et al. 1998; Scott 2000; Fernet and Hall 2004; Vral et al. 2004). In all these cases to know the *normal* response to defined doses of radiation is crucial.

The construction of in vitro dose-response curves from a healthy individual, without previous genotoxic exposures, is a useful tool in biological dosimetry. Such curves are also to be used to discover individuals departing

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significantly from the standard pattern, indicating either radiosensitivity or radioresistance. At present, these curves were constructed in the majority of studies on biological dosimetry and detection of cytogenetic lesions, such as CA or MN as biomarkers. In this context, the frequency of dicentrics in human lymphocytes is considered the most sensitive method to quantify exposures to ionizing radiation (Müller and Streffer 1991; Natarajan and Kesavan 2005), when using an appropriate in vitro calibration curve, according to the radiation quality.

In addition to the classic cytogenetic approach, the comet assay appears to be a useful tool to measure DNA breaks induced by ionizing radiation. This method provides information on a number of interesting questions in radiobiology, such as the radiosensitivity of tumor-cell populations (Olive et al. 1990; Olive 1999), and the practical advantages these have toward utilization in oncology and genetic toxicology fields (Fairbairn et al. 1995; Collins et al. 1997). Cells from peripheral blood are an ideal cell type to assess radiosensitivity in normal tissues. To obtain blood samples is considered a low invasive method, and in vitro irradiation with defined doses permits an easy determination of the incidence of DNA breakages. On the other hand, the basal level of genome damage recorded in lymphocytes is considered to reflect average genomic status of other cell types within the organism (Iarmarcovai et al. 2008; Ribeiro et al. 2008). Although the general steps of the different versions of the comet protocol are well defined, most investigators developed variants that make difficult interlaboratory comparisons of the results and, consequently, make it an impossibility to standardize the parameters that are used to quantify the assessed damage (Bocker et al. 1997).

Regarding dose-response curves, and in spite of the technical improvements and adoption of statistic software allowing comparative analysis of data collected by different laboratories, differences between laboratories usually appear. Thus, the interpretation of the exposed doses using calibration curves made in another laboratory might introduce additional uncertainty, and therefore it is recommended that, independently of the biodosimeter used, each laboratory needs to constructs its own dose-response curves (Lloyd and Edwards 1983; Lloyd et al. 1987; IAEA 2001).

The construction of an in vitro dose-response curve is supposed to determine the values of the mathematical coefficients that best fit the data points obtained in defined experiments. A dose-response curve obtained using the comet assay as a biomarker of the genetic effects induced by ionizing radiation (gamma rays) is presented. This type of curve permits one to classify individuals belonging to different groups, i.e., cancer patients or individuals with defined genotypes, as radiosensitive or radioresistant, with respect to the average values of a control population.

METHODS

Subjects and Blood Samples

Nine healthy donors (women), with no evidence of genetic disorders or previous history of exposure to known genotoxic compounds, were selected. The mean age of the group was 26.1 yr, ranging from 22 to 28 yr. Peripheral blood samples were collected from each subject (2 ml) by venipuncture using heparinized vacutainers. The whole blood was centrifuged and the supernatant removed. The cell pellet was resuspended in equal volume of RPMI 1640 medium (Gibco), and subsequently each blood sample was subdivided into six for subsequent irradiations. Before and after irradiation with gamma rays, blood samples were maintained on ice to reduce metabolic activity. Informed consent from all participants and clearance from the Ethical Committee of the University were obtained.

Irradiation Procedure

The irradiation was carried out at the Unitat Tècnica de Protecció Radiològica (UTPR-UAB) of the Universidad Autónoma de Barcelona (UAB). The irradiator was an IBL 437C, type H, number 701 (SCHERING CIS Bio International), containing up to 189 TBq (5100

curies) of cesium 137. Radiations levels at the surface were below 2.5 μSv/h, which is in compliance with the relevant international regulations.

Samples were loaded into the 3.8-L canister and installed into the unit. Proximity sensors check the closing of the door and the positioning of the canister. Time was set in sec through a digital timer. When the IBL 437C is activated from the control panel, the drum rotates 180° for exposure to radiation sources. Simultaneously the sample canister begins its rotation at 18 rpm to provide a homogeneous dose to the samples. The dose rate was 6.00 Gy min^{-1} and 5 absorbed irradiation doses were administered to each blood sample: 1, 2, 4, 6, or 8 Gy.

Alkaline Single-Cell Gel Electrophoresis Assay

The comet assay was carried out under alkaline conditions according to the method of Singh et al. (1988), with some minor modifications. Microscopic slides previously kept in ethanol–ether (2:1) and subsequently in 70% ethanol at 4°C, were used. Two solutions, one containing 0.5% normal melting agarose (NMA) (GIBCO BRL) and the other containing 0.5% low-melting agarose (LMA) (GIBCO BRL), were prepared in Ca^{2+},Mg^{2+}-free water. About 150 μl NMA was transferred onto the slide, evenly spread and kept at 60°C for 10 min to solidify. Seventy-five microliters LMA was mixed with 7.5 μl blood cell suspension (treated and control cells) and layered onto the slides, which were immediately covered with coverslips. After agarose solidification at 4°C for 10 min, the coverslips were removed and the slides were immersed overnight at 4°C in freshly lysed solution (2.5 *M* NaCl, 100 m*M* Na_2EDTA [EDTA = ethylenediamine tetraacetic acid], 10 m*M* Tris-HCl) containing 1% Triton X-100 and 10% dimethyl sulfoxide, added just before use. The slides were equilibrated in alkaline solution (1 m*M* Na_2EDTA, 300 m*M* NaOH, pH 13) for 20 min. After that, electrophoresis was carried out for 20 min at 25 V and 300 mA. Alkali treatment, as well as electrophoresis, was carried out in an ice bath. Finally, slides were neutralized by washing twice with 0.4 *M* Tris-HCl buffer (pH 7.5) for 5 min and fixed by 100% ethanol. Slides were stained with 60 μl ethidium bromide solution in distilled water (0.2 mg/ml).

Image Analysis

Observations of the cells were made at 400× magnification using a fluorescent microscope (Olympus BX50, equipped with a 515–560 nm excitation filter) connected through a Hitachi Denshi, Ltd. CCD-IRIS color video camera. The image for each individual cell was acquired immediately after opening the microscope shutter to the computer monitor, employing the Komet 5.5 Program. Cells were analyzed according to percent DNA in tail. One hundred randomly selected comet images were analyzed per treatment.

Statistical Analysis

As the comet values in lymphocytes did cover the requirements for a parametric test analysis (Kolmogorov–Smirnov test of goodness of fit), Student's *t*-test was performed for comparisons between doses. Significant differences were considered at values of $p < .001$. For the descriptive statistic, the Stat Graphics 3.0 Plus and Microsoft Office Excel 2003 software were used.

Validation Assay

In a second step, and after the construction of the curve, a validation study was carried out. New blood samples from two individuals of the studied group (selected at random) were evaluated again. Samples were irradiated with 2 and 6 Gy, and the slides were coded in a blind study. Once scored, slides were decoded and results were compared with those obtained in the overall dose-response curve.

RESULTS

Table 1 shows the basal and induced values for each of nine donors used. The percent

TABLE 1. Average Values Percentage DNA in Tail

Doses (Gy)							
Individual	0	1	2	4	6	8	*R*
1	17.63 ± 1.05	22.27 ± 1.26	24.03 ± 1.12	35.43 ± 1.07	49.78 ± 1.28	58.07 ± 1.01	.987
2	14.58 ± 1.13	12.52 ± 0.87	15.83 ± 0.77	32.56 ± 0.96	41.19 ± 1.23	55.13 ± 1.01	.96
3	14.35 ± 1.11	21.39 ± 1.17	27.90 ± 1.23	41.45 ± 1.62	50.02 ± 1.11	58.45 ± 0.91	.987
4	11.92 ± 0.86	21.13 ± 1.12	30.52 ± 1.11	35.66 ± 1.13	50.76 ± 0.97	56.50 ± 1.24	.968
5	22.15 ± 1.09	30.20 ± 1.47	29.73 ± 1.42	42.23 ± 1.53	51.41 ± 1.32	64.52 ± 1.02	.984
6	20.48 ± 1.20	25.10 ± 1.22	24.37 ± 1.17	35.08 ± 1.24	41.49 ± 1.24	52.92 ± 1.20	.975
7	17.58 ± 1.27	20.42 ± 1.11	21.48 ± 1.02	35.31 ± 1.15	50.72 ± 0.91	53.73 ± 0.84	.96
8	17.26 ± 1.00	15.98 ± 1.23	18.90 ± 0.84	32.70 ± 0.97	48.18 ± 0.95	60.10 ± 0.83	.962
9	19.88 ± 1.12	23.43 ± 0.98	26.36 ± 0.94	41.99 ± 0.73	53.02 ± 0.73	63.10 ± 0.66	.99
Mean*	17.31	21.38	24.35	36.93	48.51	58.06	.994

Note. The data show the mean of 100 cells analyzed (± standard error mean) for each individual in the range of 0 to 8 Gy. Asterisk indicates that all the induced effects are statistically significant with respect the control (0 Gy). *t*- student; $p \leq .001$

DNA in tail was the parameter used to quantify the induced damage. The percent DNA in tail is the parameter most often used in literature and it has more biological relevance than others such as the tail moment or the tail length. As observed, the induced effects increase significantly with the dose, in a direct dose-response relationship.

An overall curve was obtained by pooling together the results obtained with the nine donors. The formula underlying the curve was validated using a linear approximation. The correlation coefficient was extremely high, indicating a reliable adjustment for the mathematical model. In Figure 1 the dose-effect curve shows a mathematical description using the linear approach. The confidence intervals $(1 - \alpha) = 99\%$ were established by using the following mathematical expression:

$$(\bar{x} - z_{a/2}\frac{\sigma}{\sqrt{n}}, \bar{x} + z_{a/2}\frac{\sigma}{\sqrt{n}})$$

To probe the goodness of the dose-response curve, a validation study was carried out. In this case, new blood samples from two of nine donors previously analyzed were obtained and responses to two irradiation doses (2 and 6 Gy) were measured in a blind study. The results obtained are indicated in Table 2. As shown, the obtained values for the two donors agree with the constructed dose-response curve value, fitting such values inside the confidence interval defined (Figure 2).

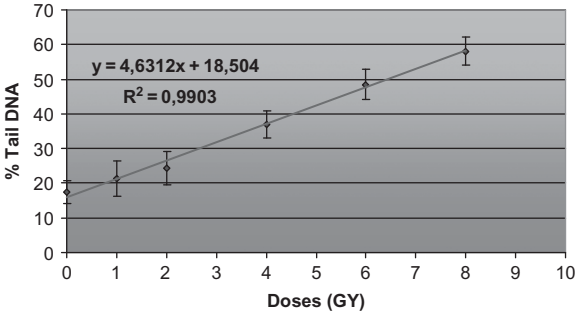

FIGURE 1. Dose-response curve for the percentage of DNA in tail. The coefficient of correlation and the adjustment to the mathematical equation is indicated (linear approach). Error bars were performed considering the deviation.

DISCUSSION

The relationship between exposure to ionizing radiation and induced genetic damage is well established by using cytogenetic damage parameters as biomarkers. Thus, calibration curves for CA and MN, with reference to ionizing radiation exposures, have already been constructed (Lloyd 1998; Schroder and Heimers 2002; Natarajan and Kesavan 2005). Although such biomarkers are unstable and, consequently, only predictive of recent exposures, the use of fluorescence in situ hybridization (FISH) techniques permits one to detect stable aberrations determining the dose received through long periods (Romm and Stephan 2004). All these biomarkers were used both as measure of in vivo irradiation and to identify individual patterns of response to

TABLE 2. Results of the Validation Study

	Dose (Gy)		Values in the curve		Limits of confidence ($\alpha = .05$)			
					2Gy		6Gy	
Donor	2 Gy	6 Gy	2 Gy	6 Gy	Lower limit	Upper limit	Lower limit	Upper limit
A	19.43 ± 9.68	51.86 ± 12.31	24.35	48.51	19.58	29.12	44.39	52.63
B	18.13 ± 9.09	50.40 ± 9.03						

Note. As indicated, the observed values for the two selected donors are between the confidence limits obtained in our dose-effect curve

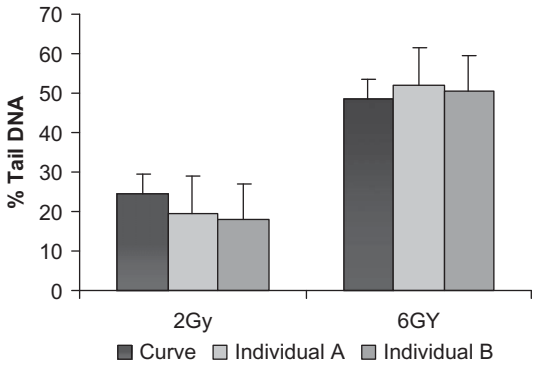

FIGURE 2. Validation study. Error bars were performed considering the deviation for each point.

in vitro irradiations. Previous studies suggested that age and gender modulate the levels of DNA damage accumulation (Dahlström Heuser et al. 2008; Mladinic et al. 2010). This bias was avoided in this study by selecting only women with a homogeneous age, ranging from 22 to 28 yr.

In addition to the preceding biomarkers, the comet assay is a new biomarker that deserves special attention. High sensitivity, together with its simplicity and low cost, offers to comet assay methodology more advantages than the classical cytogenetic assays (Lee and Steiner 2003; Guillament et al. 2004; Moller 2005). In addition, this assay was found to be particularly sensitive to the effects of ionizing radiation (Tice et al. 1990; Collins 2004). Nevertheless, several investigators claim a better standardization and calibration of the technique are required for ionizing radiation effect consequences (Bocker et al. 1997; Kassie et al. 2000; Lee et al. 2004).

Breaks and other genetic damage induced by ionizing radiation, such as those measured by the comet assay, are easily repaired (Marcon et al. 2003) and, as consequence, the existence of in vitro reliable dose-response curves does not allow their use as a biodosimeter for past exposures. Nevertheless, these curves may be used for determining the individual radiosensitivity, as deviation from a standard dose-response. Thus, obtaining good information about the normal response to a defined range of doses is crucial. It is in this case that the existence of well-constructed calibration curves demonstrates its importance.

Individual radiosensitivity is an important biomarker of the individual genomic instability, as well as a good biomarker of increased risk to the effects of genotoxic exposures, either of ionizing radiation or ofother genotoxins. In addition, such radiosensitivity might predict individual response to radiotherapy, which is an important factor when planning the doses to be applied in therapeutic protocols. New insights into the underlying molecular mechanisms of this radiosensitivity are occurring from studies assessing associations between common polymorphisms in DNA damage detection and repair genes, and development of adverse effects to radiotherapy (Fernet and Hall 2004). Independently of the molecular reasons underlying individual sensitivity, it is necessary to have reference values to determine whether a defined individual presents a particular sensitivity/resistance to ionizing radiation. In our study individual responses to increasing doses of gamma-radiation were similar; consequently, overall dose-response curves were easily obtained with a narrow interval of confidence. This confirms the goodness of the assay in measuring dose-response relationships to ionizing radiation.

In conclusion, our results show that the existence of appropriate dose-response curves in one laboratory may be used to predict the normal response in the comet assay from a particular individual to defined doses of ionizing radiation. More studies are required to determine the goodness of this notion. Thus, calibration curves with more individuals are required, and further validation studies with more individuals seem pertinent.

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