Genotoxic effects of ^{60}Co γ-rays on Chinese hamster ovary (CHO) cells

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Abstract. The aim of the present study was to evaluate the cellular radiosensitivity and radiation-induced DNA damage and repair in Chinese hamster ovary (CHO) cells. Cell survival after irradiation was assessed using the clonogenic assay. The initial, radio-induced and residual DNA damage in Chinese hamster ovary (CHO) cells exposed to ^{60}Co γ-rays were determined using the alkaline comet assay. A linear-quadratic (LQ) survival curve was observed in CHO line. Data obtained by comet assay demonstrated a linear dose-response correlation in the range of tested doses (0.3–4 Gy). The process of DNA repair was modeled by exponential equation. In addition, we found a good correlation ($R^2 = 0.995$) between clonogenic cell survival and radio-induced DNA damage.

Key words: cellular radiosensitivity • genotoxic effects • ionizing radiation • comet assay

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Introduction

Ionizing radiation is an environmental physical agent known to induce mutations and cancer [12]. Nevertheless, ionizing radiation plays an important role in the cancer treatment, radiotherapy being one of the most effective therapeutical methods for this disease [19]. The success of radiotherapy depends on the total dose administrated to the tumor, which, ideally, is conditioned by the knowledge of tumor cells' intrinsic radiosensitivity. Many studies have been carried out using a method or a combination of methods to predict tumor's response to radiotherapy. One of these is the evaluation of the survival fraction by clonogenic assay. This involves plating cells at low density in Petri dishes at least 4 h before the exposure to ionizing radiation followed by up to 4 weeks incubation, to allow colony formation. The number of colonies is directly correlated to the radiosensitivity of the investigated cells [8]. The clinical usefulness of this assay is limited because it is quite complicated, involves *in vitro* proliferation of cells and is time consuming. These limitations have stimulated research to identify methods that provide a more rapid and accurate measure of intrinsic radiosensitivity [10].

DNA damage is considered to be the main initiating event by which physical and chemical genotoxins cause genetic alterations [11]. The underlying mechanism involves genes that, directly or indirectly, control important biological functions such as cell proliferation and cell death [12]. After the direct or indirect interaction between γ-radiation and DNA molecule the following types of lesions can be identified: base damage,

DNA-DNA intrastrand and interstrand cross-links, DNA single strand breaks (SSBs), double strand breaks (DSBs) and DNA-proteins cross-links [11]. The exposure to 1 Gy causes approximately 1000 SSBs, 150 DNA-proteins cross-links and 40 DSBs [5].

The comet assay or single-cell gel electrophoresis is a sensitive method to detect DNA strand breaks, cross-links and alkali-labile sites (ALSs), as well as to evaluate the capacity of repair in individual cells [4, 17]. If alkaline conditions are applied during electrophoresis, SSBs, DSBs and ALSs are detected [18].

In the present study, we assessed the CHO cell survival using the clonogenic assay. The radio-induced DNA damage and residual DNA damage in Chinese hamster ovary (CHO) cells exposed to ^{60}Co γ-rays were measured by comet assay. The CHO cell line was chosen both for its high genetic stability, which makes it suitable for assessing DNA damage, and for its ability to form colonies. In addition, to estimate the potential of the comet assay as a predictor of cellular radiosensitivity, the relationship between clonogenic cell survival and radio-induced DNA damage was studied.

Materials and methods

Cell culture and irradiation

CHO cells that had been stored in liquid N_2 were thawed quickly and maintained in culture flasks with F12 Ham medium (Sigma) supplemented with 10% fetal calf serum, 1% penicillin and streptomycin, 1% glutamine and incubated at 37°C in the presence of 5% CO_2. Subculture was routinely performed when cells were 80–100% confluent using a solution of 0.25% trypsin.

Irradiation was carried out with ^{60}Co γ-rays (Theratron 1000) at a dose rate of 0.8 Gy/min. Exposure times were calculated for DSP, 100 cm, field 10 × 10 cm and 5 mm depth. The dosimetry of the source was confirmed using a thermoluminescence dosimeter (LiF:Mg,Ti).

For clonogenic assay, cells were seeded in 8.8 cm^2 Petri dishes. In the unirradiated control dish, 100 cells were seeded and allowed to grow 7 days before being stained. In the irradiated dishes, the number of cells seeded was between 150 cells/dish (0.3 Gy) and 2000 cells/dish (6 Gy).

For comet assay, cells were seeded at a density of 3000 cells/cm^2 in 8.8 cm^2 Petri dishes. 24 h after seeding, cells sticking to the surface of Petri dishes were irradiated with doses of 0.3, 1, 2 and 4 Gy of ^{60}Co γ-rays. Following irradiation, the cells were washed three times with PBS, treated with trypsin, harvested by centrifugation (1000 rpm, 5 min) and suspended in 250 μl of F12 medium. To evaluate the repair processes, cells were allowed to recover at 37°C and 5% CO_2 for 1 or 2 h after irradiation.

Colony formation

To assess the clonogenic potential of cells and the way it is influenced by γ-rays, irradiated cells were seeded on Petri dishes for the analysis of colony forming capacity. After 7 days at 37°C in 5% CO_2 in air and 95%

relative humidity, the medium was removed; the cells were washed with PBS and fixed with methanol for 10 min. After that, the cells were stained with Giemsa solution, and colonies exceeding 50 cells were scored. The results were expressed as survival fraction (SF), as described by Hall [6]:

$$(1) \quad SF = \frac{\text{Colonies counted in irradiated culture}}{\left[\text{Cells seeded} \times \left(\dfrac{PE}{100} \right) \right]}$$

where: PE – plating efficiency

$$(2) \quad PE = \frac{\text{Colonies counted in control culture}}{\text{Cells seeded}} \times 100$$

The survival data were fitted to the linear-quadratic model: $S = e^{-\alpha D - \beta D^2}$, were S is the survival fraction, α and β are model constants, and D is the absorbed dose in Gy.

Comet assay

The alkaline comet assay was performed according to Tice's protocol [21]. Briefly, 400 μl of 1% normal-melting-point agarose was dropped onto frosted slides, on top of which a coverglass was placed and agarose allowed to solidify. The coverglass was then removed and 85 μl of cell/agarose suspension (10 μl cell suspension and 75 μl of 0.5% low-melting-point agarose) was pipetted onto the slides and allowed to gel. The slides were gently immersed in cold lysis solution (2.5 M NaCl, 100 mM EDTA, 10 mM Tris, 1% Triton X-100 and 10% dimethyl sulfoxide added just before use) for 24 h at 4°C to lyse the cells and to allow DNA unfolding. After lysis, the slides were placed in a horizontal gel electrophoresis tank filled with fresh electrophoretic buffer (300 mM NaOH, 1 mM EDTA, pH = 13.5) and left for 20 min to allow the unwinding of DNA before electrophoresis. Electrophoresis was then carried out at 0.85 V/cm, 300 mA for 20 min. After that, the slides were rinsed three times with neutralization buffer (0.4 M Tris, pH = 7.5) and stained with 50 μl ethidium bromide (20 μg/ml).

Comets were scored using an Eclipse E-1000 fluorescence microscope (Nikon, Japan), equipped with an excitation filter of 510–560 nm and a barrier filter of 590 nm. At least 250 cells per slide were visually assigned a score on an arbitrary scale of 0–4 (i.e., ranging from 0 – undamaged, no tail to 4 – severely damaged, long diffuse tails) (Fig. 1) based on perceived comet tail length migration and relative proportion of DNA in the comet tail. The parameter used to express the DNA damage was lesion score (LS) calculated according to Collins' formula [3] and expressed as arbitrary units (a.u.):

$$(3) \quad LS_{(a.u.)} = \frac{\sum (\text{number of cells in class } X) \times (\text{class } X)}{\text{total number of cells}/100}$$

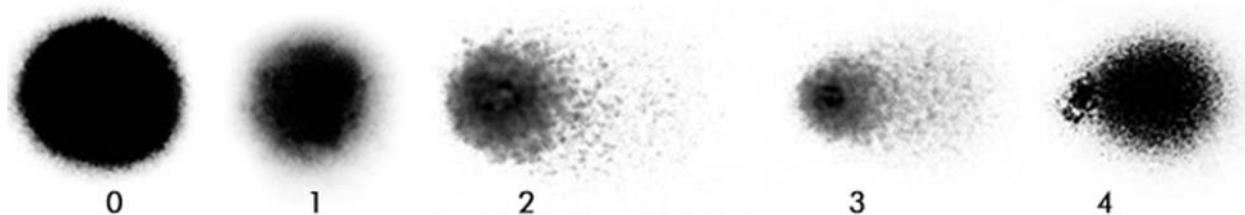where class X is from 0 (no DNA damage) to 4 (extensive DNA damage).

Fig. 1. Representations of comet images from CHO cells, comet classes 0–4.

Statistical analysis

All experiments were repeated two times with a minimum of two replicate per experiment and the data are expressed as means ± SEM. Statistical significance of differences between means was determined by Student's t-test. A p value of 0.05 or less between means was considered to be significant. All analyses were performed with GraphPad Prism program (version 5).

Results

Cell survival

Plating efficiency of non-irradiated CHO cells was found to be 57%. Figure 2 shows the survival curves of the CHO cells as functions of ^{60}Co dose, with dose plotted on a linear scale and surviving fraction on a logarithmic scale.

The curve was fitted by a linear-quadratic model:
$S = e^{[-(304\pm0.046)D-(0.010\pm0.017)D^2]}$ ($R^2 = 0.965$).

The surviving fraction at 2 Gy (SF2) was found to be 0.51 ± 0.04.

DNA damage

We examined the dose-effect relationship between ^{60}Co γ-rays irradiation and the radio-induced DNA damage level in CHO cells. A linear dose-response correlation
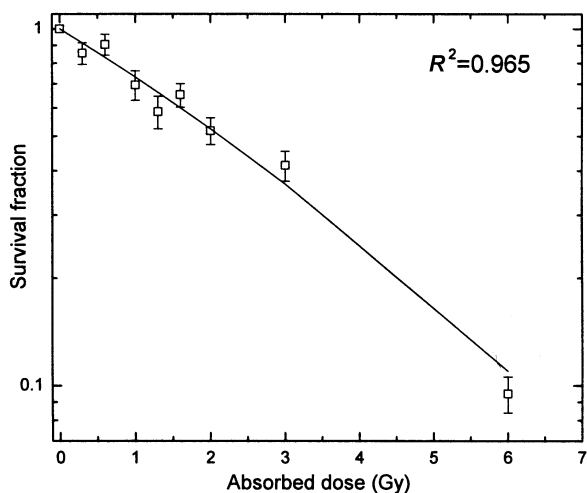

Fig. 3. DNA damage induced by ^{60}Co γ-rays in CHO cells measured by alkaline comet assay. Data points are the mean ± SEM for two independent experiments with duplicates; best linear fit is drawn. * Statistically significant difference from control at p < 0.05.

was found for the alkaline comet assay ($R^2 = 0.989$) in the range of tested doses (Fig. 3). A Student's t-test was applied for the means of each sample and a statistically significant difference (p < 0.05) between control and samples irradiated with 2 or 4 Gy was found. We also analyzed the relationship between clonogenic cell survival and lesion score, in the range of 0.3–4 Gy. The good correlation obtained ($R^2 = 0.995$) illustrate the usefulness of the comet assay to predict the radiosensitivity of individual cell lines (Fig. 4).

Fig. 2. Survival curve of CHO cells irradiated with ^{60}Co γ-rays. Data points are the mean ± SEM for two experiments with duplicates. The curve was adjusted by linear-quadratic model ($S = e^{-\alpha D-\beta D^2}$).
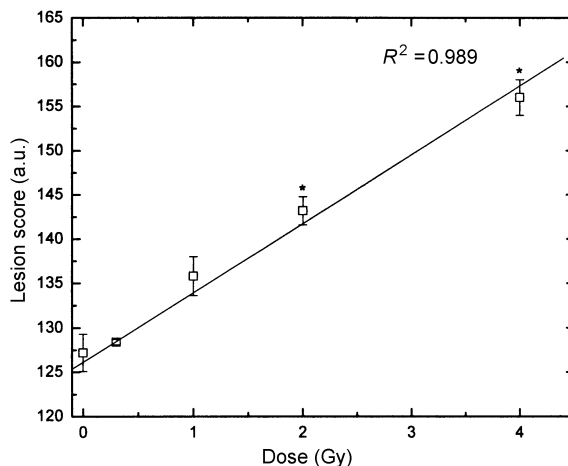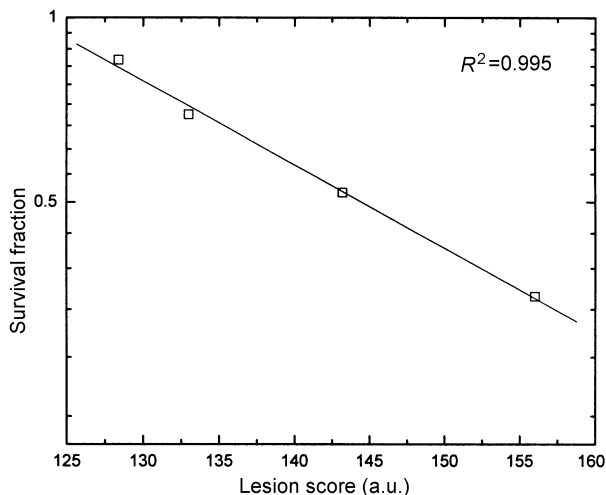

Fig. 4. The relationship between the lesion score values for radio-induced DNA damage, measured by alkaline comet assay, and the clonogenic cell survival, for CHO cells at 0.3–4 Gy.
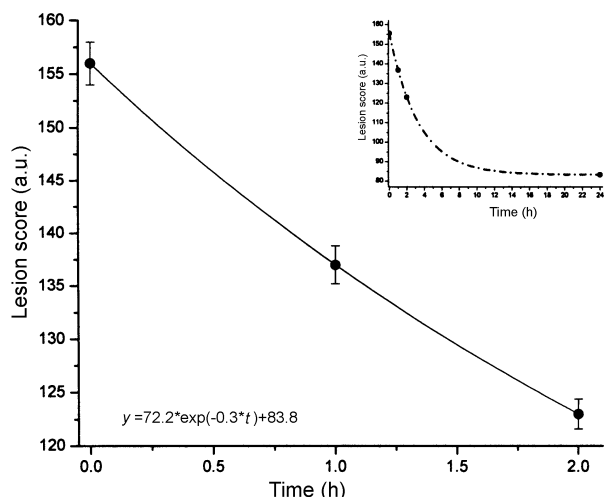
Fig. 5. Repair kinetics of DNA damage induced by 4 Gy of γ-rays in CHO cells. Data points are the mean ± SEM for two experiments with duplicates; the fit for equation $y = a^* e^{-bt} + c$ is shown. Shown inset is the estimated repair kinetics for 24 h.

DNA repair

DNA repair was assessed after 1or 2 h incubation of the irradiated cells at 37°C and 5% CO_2 and calculated by the lesion score expressed in arbitrary units. Figure 5 shows the capacity of cells to repair the DNA damage induced by 4 Gy of γ-rays, as well as the kinetics of this process.

The repair process can be described by the exponential model:

$$(4) \qquad Y = a^* e^{-bt} + c$$

where *a* is the initial repairable damage, *b* is the repair coefficient and *c* is the residual non-repairable damage calculated by extrapolation [14, 24]. If 1 h after irradiation the level of DNA damage is reduced by aproximatively 12%, after 2 h this level is reduced by 22%. There was a statistically significant difference between the value obtained immediately after irradiation and those obtained after 1 h (p < 0.05), and especially 2 h after exposure (p < 0.01).

Discussion

The main objective of the present study was to evaluate the genotoxicity of γ-rays on a CHO cell line, using clonogenic assay and comet assay. Several studies found a linear-quadratic distribution for the cell survival after irradiation with γ-rays, as well as a linear relationship between the level of radio-induced DNA damage and the absorbed dose [1, 2, 7, 10, 12, 23]. The fitted cell survival curve for the CHO cells was linear quadratic in the present study (Fig. 2), which is consistent with other previous studies [1, 12].

Although clonogenic assay represents the "golden standard" for evaluation of cellular radiosensitivity, the long time required for colony formation (2–4 weeks) reduces clinical usefulness of this assay as a predictive assay for radiation response. In these circumstances, it is essential to find an alternative method to estimate the cellular radiosensitivity. Comet assay is a cheap, rapid and sensitive method for the evaluation of radio-induced DNA lesions and their repair in individual cells [17]. The low number of cells and the short time required to obtain the results make this method an ideal tool for clinical applications [10]. Using the alkaline version of comet assay, we found that DNA damage was induced in a linear dose-dependent way by [60]Co γ-rays (Fig. 3). In addition, an inverse correlation between cell survival (as a measure of cellular radiosensitivity) and radio-induced DNA lesions was found (Fig. 4). This suggests that comet assay could be used as a predictive test for cellular response to radiotherapy. Similar results have been reported in other studies, performed both on human [8, 10, 15] and animal cell lines [7, 23], using alkaline or neutral versions of the comet assay. This correlation was found not only for radio-induced DNA lesions [7–10, 15] but also for the residual lesions evaluated at different time moments [9, 10, 23]. Moneef *et al.* [10], in a study performed on six human bladder cell lines, obtained better than that a correlation between SF2 and radio-induced DNA lesions (immediate after the exposure) in comparison between SF2 and residual lesions after 15 and 30 min. The author suggests the usefulness of using the radio-induced DNA lesions as a predictive factor for the cell survival of the studied cell lines.

DNA repair process plays a vital function in protecting normal cells from the effects of radiation, including cancer development [20]. The kinetics of radio-induced DNA repair of CHO cells seems to be biphasic: a rapid and a slow phase of the repair process could be described (Fig. 5 inset). The fast DNA repair component can be explained by the fact that most SSBs are repaired very quickly (in the first 3 h after irradiation) [13, 16]. The slow DNA repair component may be due to some double strand breaks repair processes [22]. This is consistent with some previous studies [2, 10, 15, 22, 24]. However, compared to other cell lines [10, 15] the DNA repair kinetics in CHO cells is more slowly. Similar repair kinetics was observed after 5 Gy irradiation of C3H10T1/2 cells with [137]Cs γ-rays [2].

In conclusion, we found a linear dose-response correlation between γ-irradiation dose and radio-induced DNA damage. In addition, we described the repair process with an exponential model that includes information about the amount of the repairable damage and the value corresponding to the amount of residual non-repairable DNA damage. Overall, our study illustrates that the genotoxic effects of γ-radiation can be identified by clonogenic assay, as well as comet assay. Moreover, the correlation found between survival fraction and radio-induced DNA damage shows the possibility of using the alkaline comet assay as a method to evaluate the cellular radiosensitivity. Further studies are required to confirm the validity of this method as a predictive test for cellular response to ionizing radiation (radiotherapy).

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