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CYTOGENETIC AND CYTOMOLECULAR DELAYED DAMAGE INDUCED IN HUMAN FIBROBLASTS BY LOW X-RAY DOSES

PONZINIBBIO MV[1-3], CRUDELI C[1-2], PERAL GARCÍA P[1] AND SEOANE A[1-3]

[1]IGEVET (Centro de Investigaciones en Genética Básica y Aplicada) Facultad de Ciencias Veterinarias. Universidad Nacional de La Plata. Calle 60 y 118, CC 296 B1900AVW
La Plata, Argentina. aseoane@fcv.unlp.edu.ar
[2]ANPCyT (Agencia Nacional de Promoción Científica y Tecnológica), Argentina
[3]CONICET (Consejo Nacional de Investigaciones Científicas y Técnicas), Argentina

ABSTRACT

During the last decade several reports have provided evidence on the non-targeted effects of ionizing radiation. In this study, we have analyzed the delayed DNA damage induced by ionizing radiation in MRC-5 Human fibroblasts cultured *in vitro*.
MRC-5 cells were irradiated with 10 and 50 milligrays (mGy) and analyzed immediately after exposure, and at passages 6 and 12 post-radiation by means of comet assay, micronucleus analysis and γ-H2AX foci formation. Cytogenetic and cytomolecular damage was analysed at every sample point comparing irradiated and control cells. No significant damage differences were found among samples from all passages of treatments.
Our results suggest that even at low doses, ionizing radiation could induce delayed damage. Comet assay, micronucleus analysis and γ-H2AX focus analysis showed to be useful techniques to detect DNA damage and might be also sensitive for the study of delayed events as genomic instability.

Keywords: ionizing radiation, low radiation dose, DNA damage

RESUMEN

Durante la última década se ha ido acumulando evidencia acerca de los efectos no específicos de las radiaciones ionizantes. En el presente trabajo analizamos el daño inducido por radiación ionizante en el ADN de la progenie de fibroblastos humanos de la línea MRC-5 cultivados *in vitro*.
Se irradiaron las células con 10 y 50 miligrays (mGy) y se analizaron inmediatamente después de la exposición, y en los pasajes 6 y 12 post-irradiación a través del ensayo cometa, análisis de micronucleos y conteo de focos de γ-H2AX.
Se observó daño citogenético y citomolecular en todos los puntos muestrales analizados cuando comparamos las células irradiadas con las correspondientes al control negativo. No hubo diferencias significativas al comparar las muestras de cada tratamiento tomadas en los diferentes pasajes.
Los resultados obtenidos sugieren que las dosis muy bajas de radiación ionizante pueden inducir daño no sólo en las células expuestas sino también en su progenie. El ensayo cometa, análisis de micronúcleos y conteo de focos de γ-H2AX son consideradas técnicas útiles para detectar daño en el ADN y según nuestros resultados, podrían emplearse para analizar eventos retrasados en el tiempo.

Palabras clave: radiaciones ionizantes, dosis bajas de radiación, daño en el ADN

Introduction

During the last decade several reports have provided evidence on the non-targeted effects of ionizing radiation, which could be quite significant for evaluating the consequences of radiation exposure, particularly at low doses (Morgan, 2003). Models based on the existing paradigm of radiation biology have been usually employed to assess the risk of low dose exposure. Morgan (2003) claims that the central dogma of radiation action should be re-evaluated because non-targeted effects amplify the biological effectiveness of a given radiation dose. Radiation-induced genomic instability occurs in cells descending from irradiated ones (Morgan et al., 1996; Little et al., 1998; Mothersill and Seymour, 1998; Lorimore, 2003; Little et al., 2006). Increased frequency of a variety of genetic end-points such as micronuclei, apoptosis, mutagenesis, and chromosomal instability, was observed in the progeny of surviving radiation-exposed cells (Little, 2003; Ponnaiya et al., 1997; Kadhim et al., 1995; Limoli et al., 1997). These endpoints are characterized by the delayed expression in several generations and are readily induced by radiation in those systems in which they occur (Baverstock, 2000).

In the present work, radiation-induced damage was analyzed in the progeny of X-ray-treated human fibroblasts by means of comet assay, micronucleus analysis and γ-H2AX foci formation.

Material and methods

Cells

MRC-5 cells obtained from the American Type Culture Collection (Manassas, VA, USA) were cultured in MEM medium (Gibco BRL, Grand Island, NY, USA) supplemented with 10% fetal bovine serum (Notocor Laboratories, Córdoba, Argentina) and antibiotics (50 IU penicillin and 50 μg/ml streptomycin; Bagó Laboratories, Buenos Aires, Argentina) in a 5% CO^2 humidified atmosphere. Cells were cultured in Falcon T-25 (Nunc, Denmark) with 10 ml culture medium.

Experimental design

Radiation treatments were performed when cells were 90-95% confluent. They were carried out on ice in order to avoid the repair of strand breaks during the procedure. Following our previous research experience (Güerci et al., 2003; 2004; Seoane et al., 2007) and dosimetry reported elsewhere (Barquinero et al., 1993; Paz y Miño et al., 1995; Balakrishnan and Rao, 1999; Cardoso et al., 2001; Cavallo et al., 2002), two X-ray doses were employed: 10 and 50 milligrays (mGy).

X-ray radiation equipment used was from Dental San Justo Company (Buenos Aires, Argentina) operated at 65kV and 5 mA. Doses were determined by dosimeter Keithley Digital 35617 EBS microchamber PTW N 2336/414 (C-Com Industries, Robertville, MO, USA) at 50 mGy/min dose rate. Samples were irradiated from above through the medium and exposure times were 12 and 60 seconds for 10 and 50 mGy respectively.

After treatment cells were tripsinized, resuspended, and divided into four fractions. One was cultured on coverslips for γ-H2AX focus analysis, other was cultured in T-25 flasks for micronucleus analysis, aliquots were obtained for comet assay, and the last fraction was maintained in culture for 12 passages. A control group remained untreated.

Control and irradiated populations were assayed immediately after radiation and at 6 and 12 passages post-radiation. Doubling time of MRC-5 cells under these culture conditions was periodically checked using the bromodeoxyuridine technique (BrdU); it varied between 24-26 h. Each experiment was repeated twice. Average values are shown in Tables. Blind analysis was carried out by one investigator.

Comet assay

Single cell gel electrophoresis was

performed using the alkaline technique described by Singh (1988) with some modifications (Tice and Strauss, 1995). Briefly, slides were covered with a first layer of 180 µl 0.5% normal agarose (Carlsbad, Ca, USA). An amount of 75 µl 0.5% low melting point agarose (Carlsbad, Ca, USA) was mixed with approximately 15,000 cells suspended in 15 ìl and layered onto the slides, which were then immediately covered with coverslips. After agarose solidification at 4°C for 10 min, coverslips were removed and slides were immersed overnight at 4°C in fresh lysis solution. The slides were balanced in alkaline solution for 20 min. Electrophoresis was carried out for 30 min at 25 V and 300 mA (1.25 V/cm). Afterwards, slides were neutralized by washing them three times with Tris buffer (pH 7.5) every 5 min and subsequently washed in distilled water. Slides were stained with 1/1000 SYBR Green I solution (Molecular Probes, Eugene, Oregon, USA) (Ward and Marples, 2000). Two hundred randomly selected comet images were assessed per treatment. Data were statistically analyzed using the X^2-test with the Statgraphics® 5.1 software.

Image analysis

A fluorescent microscope (Olympus BX40 equipped with a 515-560 nm excitation filter) connected to a Sony 3 CCD-IRIS Color Video Camera was used for scoring at 400x magnification.

Based on the extent of strand breakage, cells were classified according to their tail length in five categories, ranging from 0 (no visible tail) to 4 (comet head still detectable but most of DNA in the tail) (Olive, 1999; Collins, 2004). DNA damage from the comets was measured following Collins method (2004). When 100 comets are scored, and each comet is assigned a value of 0 to 4 according to its class, the total score for the sample gel will be between 0 to 400 "arbitrary units". Visual scoring (arbitrary units) is rapid as well as simple and shows very close agreement with computer image analysis (DNA percentage in tail) (Collins, 2004).

Immunofluorescence analysis

A modified method of Rothkamm and Lobrich (2003) was applied. Cells grown on coverslips were fixed in 100% methanol for 30 min and 100% acetone at 4ºC for 1 min, washed in PBS for 3 x 10 min, and permeabilized for 5 min on ice in 0,2% Triton X-100. Samples were blocked in PBS with 1% BSA for 3 x 10 min at room temperature. The coverslips were incubated with anti-γ-H2AX antibody (Trevigen, Gaithersburg, MD, USA) for 1 h, washed in PBS, 1% BSA for 3 x 10 min, and incubated with Alexa Flour 488-conjugated goat anti-rabbit secondary antibody (Molecular Probes, Eugene, Oregon, USA) for 1 h at room temperature. Cells were washed in PBS for 4x10 min and mounted by using Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA) with 0.5 µg/ml propidium iodide.

Foci were eye-counted ((by eye)) at 400x magnification using a fluorescent microscope (Olympus BX40 ((equipped)) with a 515-560nm excitation filter). CHO cells were analysed 50 min after radiation and MRC-5 cells, 30 min post-radiation because the highest frequency of foci was scored at that time (data not shown). At least 300 cells were analyzed per experimental point. None of the cells analyzed showed more than two foci. Cells with foci frequency are shown in Tables. Data were statistically analyzed using the X^2-test with the Statgraphics® 5.1 software.

Micronucleus test

The cytokinesis-blocked micronucleus assay was modified from Fenech and Morley (1985). Cells were cultured as monolayers during two cell cycles. At the end of the first cycle, B-cytochalasin (3 mg/ml final concentration) (Sigma, St. Louis, MO, USA) was added. Cells were then removed by trypsinization and agitation. The cell

suspension was centrifuged and the pellets resuspended in 5 ml fixative methanol acetic acid (3:1). Cells were washed three times with fresh fixative methanol acetic acid, resuspended, dropped onto clean slides, and stained with 4% Giemsa for 10 min. One-thousand binuclear cells were analyzed per experimental point. Fenech and coworkers (2003) scoring criteria were followed for micronuclei determination. The X^2- test with the Statgraphics® 5.1 software was used for statistical analysis.

Results

Tables I to III summarize damage degree analysis of comet assay. Damage index measured by "arbitrary units" is represented in Figure 1. Treatment with 10 mGy induced higher DNA damage than control at passages 6 and 12 ($p<0.001$ and $p<0.01$ respectively). Significant increase in damage index ($p<0.001$) was observed for 50 mGy dose when compared with control at all sample points analyzed. There were no significant differences between passages for all treatments; cells treated with 10 mGy showed higher damage at passage 6 and those treated with 50 mGy showed higher damage at passage 12.

Passage	DNA damage level (%)				
	Degree 0	Degree 1	Degree 2	Degree 3	Degree 4
0	86.03	7.98	4.74	0.50	0.75
	(0.34)	(0.27)	(0.21)	(0.07)	(0.08)
6	89.56	5.38	3.16	1.58	0.32
	(0.30)	(0.22)	(0.17)	(0.12)	(0.05)
12	89.07	6.05	3.95	0.70	0.23
	(0.31)	(0.23)	(0.19)	(0.08)	(0.04)

Table I. Mean frequencies (average ± standard error) of damage degrees in unexposed MRC-5 cells

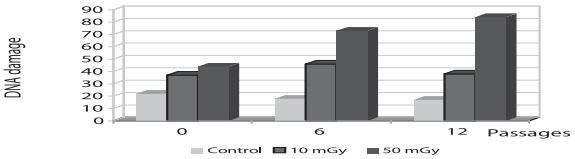

Table II. Mean frequencies (average ± standard error) of damage degrees in 10 mGy irradiated MRC-5 cells

Passage	DNA damage level (%)				
	Degree 0	Degree 1	Degree 2	Degree 3	Degree 4
0	61.92	12.81	12.45	5.69	6.76
	(0.48)	(0.33)	(0.33)	(0.23)	(0.25)
6	54.44	24.53	16.36	2.57	2.10
	(0.49)	(0.43)	(0.36)	(0.15)	(0.14)
12	61.43	13.33	12.38	5.71	7.14
	(0.48)	(0.33)	(0.32)	(0.23)	(0.25)

Table III. Mean frequencies (average ± standard error) of damage degrees in 50 mGy irradiated MRC-5 cells

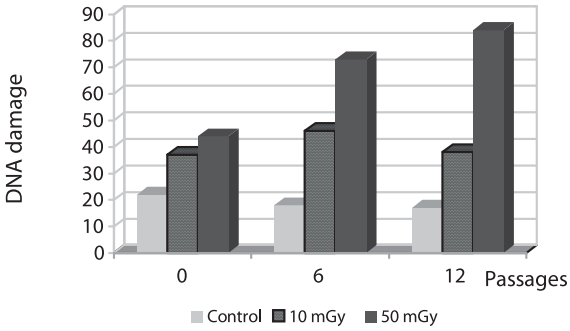

Table IV shows data from γ-H2AX focus and micronucleus analyses. The frequency of cells with foci for the two radiation doses used was higher (but not significant) than that of control when the analysis was carried out immediately after exposure and at passages 6 and 12. In addition, cells at passage 6 showed higher damage than the others, although differences were not statistically significant. A value of 0.05 foci per cell means that 1 cell in 20 contains a focus (Rothkamm and Lobrich, 2003). Taking this into account, results presented here vary from 0.134 to 0.158 (cells with 1 or 2 foci respectively) for 10 mGy dose, and from 0.194 to 0.212 for 50 mGy dose.

Micronucleus frequency of cells treated with 50 mGy showed significant increase with respect to control at day 12 ($p<0.005$). All other frequencies showed no significant differences with control. When damage observed immediately after exposure was compared with that observed at passages 6 and 12 for each treatment, results indicated that cells scored at passage 6 showed higher damage than those observed immediately after exposure (except for ID in cells treated with 50 mGy). No significant differences were found between each treatment analyzed at different passages.

Treatment	Passages					
	0	6	12	0	6	12
	Cells with γ-H2AX foci (%)			Cells with micronuclei (‰)		
Control	3.33	5.63	3.91	5.0	6.0	8.0
	(0.17)	(0.23)	(0.19)	(0.07)	(0.07)	(0.08)
10 mGy	4.17	6.27	5.19	9.0	22.0	15.0
	(0.19)	(0.24)	(0.22)	(0.09)	(0.10)	(0.12)
50 mGy	6.05	9.32	5.94	16.0	34.0	25.0
	(0.23)	(0.29)	(0.23)	(0.12)	(0.12)	(0.15)

Table IV. Mean frequencies (average ± standard error) of unexposed and irradiated MRC-5 cells containing γ-H2AX foci and micronuclei.

Discussion

A variety of genotoxic and cytotoxic agents may potentially affect the stability of the cellular genome. Ionizing radiation is an ubiquitous environmental physical agent that has proved to exert DNA-damaging effects. Radiobiology interpretation accepts that after exposure to DNA damaging agents, mutation frequency increases rapidly at the beginning and declines to background levels when DNA damage repair is completed. This concept on mutagenesis has been challenged in recent years after several findings on radiation-induced genomic instability (Little, 2003; Morgan, 2003). Genomic instability could be considered as a radio-response in which the stability of genomic integrity has been affected. The endpoints characteristic of genomic instability provide evidence for a prolonged disruption to cellular homeostasis that perpetuates the memory of past insult over several cellular generations (Limoli, 2003; Niwa, 2006).

The present work has analysed delayed DNA damage at 6 and 12 passages. It is assumed that stochastically induced biological effects are the result of damage to DNA sequences that fix and replicate in all progeny (Baverstock, 2000). Our results agree with the hypothesis of a process by which initial DNA damage becomes permanent or is memorized by the surviving cells. In this sense, results agree with Susuki (2003; 2006) and Little (2003) who considered unlikely that DNA strand breaks are inherited through many cell divisions in normal cells and that radiation enhances the frequency with which genetic changes arise spontaneously in the cell population derived from the irradiated cell. In order to explain this mechanism, Raser and O'Shea (2005) proposed that radiation induces certain conditions that stimulate the production of reactive oxygen species (ROS) generating a genotoxic environment and promoting the appearance of phenotypes associated with genomic instability. The persistence of ROS indicates a continuous turnover of oxidative species perpetuating a source of damage over time that could account for the delayed damage observed (Morgan, 2003; Limoli et al., 2003).

Results presented here showed the genotoxic effect observed in MRC-5 cells at all steps analyzed, suggesting the early appearance and maintenance of the unstable phenotype. Values from γ-H2AX focus analysis observed immediately after exposure are similar but slightly lower than those reported by Rothkamm and Lobrich (2003) who scored 0.3 foci per 10mGy-treated MRC-5 cell.

The capacity of radiation to induce genomic instability depends, to a large extent, on its quality and dose. There appears to be a low dose threshold effect beyond which no additional genomic instability is induced. Suzuki (2006) has calculated that the number of phosphorylated H2AX foci corresponding to 0.5-1 Gy equivalent doses is sufficient to activate a DNA damage checkpoint. In this study, we have found that doses from 10 to 50 mGy can induce delayed damage in MRC-5 cells suggesting that the threshold mentioned would be lower than 10 mGy. Although the mechanisms underlying ionizing radiation effects are not completely understood, our results show that low X-ray doses could induce genetic damage in the progeny of irradiated cells.

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