ORIGINAL ARTICLE

ACTA RADIOLOGICA

Low-dose radiation employed in diagnostic imaging causes genetic effects in cultured cells

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Background: Exposure to environmental, diagnostic, and occupational sources of radiation frequently involves low doses. Although these doses have no immediately noticeable impact on human health there is great interest in their long-term biological effects.

Purpose: To assess immediate and time-delayed DNA damage in two cell lines exposed to low doses of ionizing radiation by using the comet assay and micronucleus test, and to compare these two techniques in the analysis of low-dose induced genotoxicity.

Material and Methods: CHO and MRC-5 cells were exposed to 50 milliSievert (mSv) of ionizing radiation and assayed immediately after irradiation and at 16 or 12 passages post-irradiation, respectively. Comet assay and micronucleus test were employed.

Results: The comet assay values observed in 50 mSv-treated cells were significantly higher than in the control group for both sample times and cell lines ($P < 0.001$). Micronuclei frequencies were higher in treated cells than in the control group ($P < 0.01$, CHO cells passage 16; $P < 0.05$, MRC-5 cells immediately after exposure; $P < 0.01$ MRC-5 cells passage 12). Correlation analysis between the two techniques was statistically significant (correlation coefficient 0.82, $P < 0.05$ and correlation coefficient 0.86, $P < 0.05$ for CHO and MRC-5 cells, respectively). Cells scored at passages 12 or 16 showed more damage than those scored immediately after exposure in both cell lines (no statistically significant differences).

Conclusion: Cytomolecular and cytogenetic damage was observed in cells exposed to very low doses of X-rays and their progeny. A single low dose of ionizing radiation was sufficient to induce such response, indicating that mammalian cells are exquisitely sensitive to it. Comet and micronucleus assays are sensitive enough to assess this damage, although the former seems to be more efficient.

Key words: DNA damage; genomic instability; ionizing radiation; micronuclei

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Owing to its ability to deposit energy within the cells, ionizing radiation has some unique characteristics as a mutagenic and carcinogenic agent and there is no doubt about the risk that the exposure to high doses of ionizing radiation poses for human health (1, 2). However, exposure to environmental, diagnostic, and occupational sources of radiation frequently involves low doses. Although these doses have no immediately noticeable impact on human health, there is great interest in their long-term biological effects (3). Clearly, it is important for the sake of the general population to investigate the effects of low-dose exposure in a range close to that found in occupationally exposed individuals.

Evidence from several studies conducted among radiation-exposed individuals or cells showed significant increases in the frequency of chromosomal aberrations or micronuclei (4–12). In addition, radiation-induced DNA damage in individual cells was usually evaluated by the single cell electrophoresis or comet assay, which is commonly used as it is considered to be simpler and faster than other assays (10, 13–16). Comparative investigations between the comet assay and the micronucleus test using radiation or chemicals as mutagens have been carried out (17–21). In this sense, Maluf et al. (22) reported that the micronucleus frequency was significantly correlated with the comet assay damage index; and He et al. (23) found that the DNA damage induced by 0.05 Grays (Gy) (50 mGy) of ionizing radiation could be detected by means of the comet assay but the micronucleus rate did not increase significantly until a radiation dose of 0.25 Gy was delivered.

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On the other hand, beyond the DNA damage arising as a result of the direct impact of the radiation in the genetic material, it is widely accepted that there are other consequences associated with low-dose radiation. Genomic instability can be manifested as elevated rates of heritable changes in the progeny of irradiated cells and has a significant value in the risk assessment of low-dose radiation. It can be measured as chromosomal aberrations, micronucleus formation, gene mutations, and microsatellite instabilities, as well as other end points (24–26). Elevated rates of micronucleated cells were observed in the progeny of primary human fibroblasts and V-79 cells irradiated with X-rays or γ-particles (27–29). On the contrary, no evidence of persistent transmissible genomic instability was found in a study of blood lymphocytes of radiation workers with internal deposits of plutonium (30) and in normal diploid human fibroblasts (AG1521A) that survived after exposure in G0 to low- and high-LET radiation (31).

The purpose of the present study was to assess genetic instability and heritable damage (predisposition to DNA-DSB, -SSB, and alkaline-labile sites) in two cell lines exposed to low doses of ionizing radiation by using the micronucleus test and alkaline comet assay, as well as to compare the two techniques as tools for the analysis of low-dose induced genotoxicity.

Material and Methods

Cells

CHO and MRC-5 cells were obtained from the American Type Culture Collection (ATCC, Manassas, Va., USA). Cells were cultured in Ham's F10 medium (Gibco BRL, Grand Island, N.Y., 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 humidified atmosphere with 5% CO_2. Cells were cultured in Falcon T-25 (Nunc, Roskilde, Denmark).

Experimental design

Irradiation treatments were performed with non-dividing confluent cell cultures. They were carried out on ice to prevent the repair of strand breaks during the procedure. The radiation dose delivered was 50 mSv (≈ 50 mGy) of ionizing radiation, taking into account previous experiences in our laboratory (9, 10, 12) and the dosimetry reported in epidemiological exposures (4–6, 22, 23, 32).

The irradiation equipment was provided by Dental San Justo Company (Buenos Aires, Argentina) and operated at 65 kV and 5 mA. Doses were determined by a dosimeter (Keithley Digital 35617 EBS microchamber PTW N 2336/414; C-Com Industries, Robertville, Mo., USA) and administered at a dose rate of 50 mSv/min. Radiation was delivered from above through the medium and exposure times were 60 s. For the irradiation treatment, 10 ml of fresh medium was placed on the attached cells to prevent the presence of detached cells.

After treatment, cells were trypsinized, resuspended, and divided into three fractions. One was cultured in T-25 flasks for micronucleus analysis, aliquots were obtained for comet assay, and the last fraction was kept in culture for 12 (MRC-5) or 16 passages (CHO). MRC-5 cells failed to reach more than 12 passages in our laboratory conditions. A control group remained untreated.

Control and irradiated populations were assayed immediately after irradiation and at 12 or 16 passages post-irradiation. The doubling time for CHO and MRC-5 cells under these culture conditions was periodically verified in the laboratory using a bromodeoxyuridine technique (BrdU); it varies between 12 and 15 h for CHO cells and between 24 and 26 h for MRC-5 cells. Each experiment was repeated twice and average values are shown in Tables 1–3. Blinded analysis was carried out by one investigator.

Comet assay

Single cell gel electrophoresis was performed employing the alkaline version described by Singh et al. (33) with some modifications (34). Briefly, slides were covered with a first layer of 180 μl of 0.5% normal agarose (Invitrogen, Carlsbad, Calif., USA). Then 75 μl of 0.5% low melting point agarose (Invitrogen,Carlsbad, Calif., USA) was mixed with approximately 15 000 cells suspended in 25 μl of fresh culture medium and laid onto the slides, which were then immediately covered with coverslips. After agarose solidification at 4°C for 10 min, coverslips were removed and the slides were immersed overnight at 4°C in fresh lysis solution. The slides were equilibrated in alkaline solution for 20 min. Electrophoresis was carried out for 30 min at 25 V and 300 mA (1.25 V/cm). Afterwards, they were neutralized by washing three times with Tris buffer (pH 7.5) every 5 min and subsequently washed in distilled water. Next, they were stained with 1/1000 SYBR Green I solution (Molecular Probes, Eugene, Oreg., USA) (35). A total of 200 randomly selected comet images were analyzed per treatment. Data were statistically analyzed using the χ^2 test with Statgraphics® 5.1 software (Manugistics Group Inc., Rockville, Md., USA).

Image analysis

Scoring was performed at ×400 magnification with a f luorescent microscope (Olympus BX40; Olympus Optical Co. Ltd, Tokyo, Japan, equipped with a 515–560 nm excitation filter) connected through a Sony 3

Table 1. Mean frequencies (average ± standard error) of damage degrees and index damage in CHO cells.

		DNA damage (%)					
Treatment	Passage	Degree 0	Degree 1	Degree 2	Degree 3	Degree 4	Index damage
Non-irradiated	0	91.77 (0.27)	4.53 (0.20)	3.29 (0.17)	0.41 (0.06)	–	12.35
	16	91.76 (0.27)	6.27 (0.24)	1.18 (0.11)	0.78 (0.09)	–	10.98
50 mSv- irradiated	0	61.97 (0.48)	26.92 (0.44)	9.40 (0.29)	0.43 (0.06)	1.28 (0.11)	52.14
	16	59.39 (0.49)	25.67 (0.44)	11.88 (0.32)	1.53 (0.12)	1.53 (0.12)	60.15

CCD-IRIS color video camera. Based on the extent of strand breakage, cells were classified according to their tail length in five categories, ranging from grade 0 (no visible tail) to grade 4 (still a detectable head of the comet but most of the DNA in the tail) (35, 36) (Fig. 1). The method of COLLINS (36) was used to compute DNA damage from the comet. Index damage (ID) was obtained: if 100 comets are scored, and each comet is assigned a value of 0–4 according to its category, the total score for the sample gel will range between 0 and 400 "arbitrary units." Visual scoring (arbitrary units) is rapid as well as simple, and this method and computer image analysis (percentage DNA in tail) are in very close agreement (36).

Micronucleus test

The cytokinesis-block micronucleus assay was modified from FENECH et al. (37). Cells were cultured as monolayers during two cell cycles, 30 h and 48 h for CHO and MRC-5 cells, respectively. At the end of the first cycle, B-cytochalasin (3 μg/ml final concentration) (Sigma, St Louis, Mo., USA) was added to the cultures. Cells were then removed by trypsinization and agitation. The cell suspension was centrifuged and the pellet was resuspended in 5 ml of fixative (methanol:acetic acid 3:1). The cells were washed with fresh fixative three times, resuspended, dropped onto clean slides, and stained with 4% Giemsa for 10 min. One thousand binucleated cells were analyzed per experimental point. FENECH et al. (37) scoring criteria for micronuclei determinations were used. The χ^2 test with Statgraphics® 5.1 software was used for statistical analysis.

Results

CHO cells

Comet results expressed as damage degrees and ID are summarized in Table 1. The comet assay values scored in 50 mSv-treated cells were significantly higher than in the control group, not only immediately after exposure but also at the end of the experiment ($P < 0.001$). Micronuclei results are summarized in Table 2. Micronuclei frequencies were higher in treated cells than in the control group, but these differences were statistically significant only in cells analyzed at passage 16 ($P < 0.01$).

MRC-5 cells

Comet results expressed as damage degrees and ID are summarized in Table 3. The comet assay values observed in 50 mSv-treated cells were significantly higher than in the control group at both sample times ($P < 0.001$). Micronuclei results are summarized in Table 2. Micronuclei frequencies were significantly higher in treated cells than in the control group immediately after exposure ($P < 0.05$) and at passage 12 ($P < 0.01$).

Correlation analysis was performed to compare results obtained with the two techniques. Comparison showed a correlation coefficient of 0.82 ($P < 0.05$) for CHO cells and 0.86 ($P < 0.05$) for MRC-5 cells. When sample times were compared, cells scored at passages 12 or 16 showed more damage than that observed immediately after exposure in both cell lines, but these differences were not statistically significant.

Discussion

When cells are exposed to low doses of ionizing radiation, double-strand break formation is one of the most important kinds of damage observed (38). If the repair is either wrong or not possible, a cell is meant to die or remain damaged (26, 39). In our study, both cytogenetic instability (micronucleus induction) and heritable damage (predisposition to DNA lesions), were detected in hamster and human cells.

Table 2. Mean frequencies (average ± standard error) of micronuclei (‰) in CHO and MRC-5 cells.

	Passages			
	CHO cells		MRC-5 cells	
Treatment	0	16	0	12
Non-irradiated	6.0 (0.05)	5.0 (0.07)	5.0 (0.07)	8.0 (0.08)
50 mSv- irradiated	11.0 (0.07)	21.0 (0.10)	16.0 (0.12)	25.0 (0.15)

Table 3. Mean frequencies (average ± standard error) of damage degrees and index damage in MRC-5 cells.

		DNA damage (%)					
Treatment	Passage	Degree 0	Degree 1	Degree 2	Degree 3	Degree 4	Index damage
Non-irradiated	0	86.03 (0.34)	7.98 (0.27)	4.74 (0.21)	0.50 (0.07)	0.75 (0.08)	21.95
	12	89.07 (0.27)	6.05 (0.24)	3.95 (0.11)	0.70 (0.09)	0.23 (–)	16.98
50 mSv- irradiated	0	61.92 (0.48)	12.81 (0.33)	12.45 (0.33)	5.69 (0.23)	6.76 (0.25)	82.14
	12	61.43 (0.48)	13.33 (0.33)	12.38 (0.32)	5.71 (0.23)	7.14 (0.25)	83.81

Results found with micronuclei analysis immediately after exposure are consistent with those obtained in individuals exposed to similar doses (4–8, 15). On the contrary, KASHINO et al. (11) found induction of micronuclei in xrs-5 cells when they were irradiated at 0.05 Gy (50 mGy ≈ 50 mSv) of ionizing radiation but not in EM9 and CHO cells, and HE et al. (23) observed that the micronucleus rate did not increase significantly until a radiation dose of 0.25 Gy was delivered. Our findings with the comet assay are consistent with those of other authors (40, 41) who found DNA damage after exposure to doses of 50 mGy (≈ 50 mSv) of ionizing radiation. ASAITHAMBY & CHEN (42) recently provided evidence that the number of DNA-DSB induced by γ-irradiation increases linearly with increasing doses throughout a range from 5 mGy to 1 Gy. Altogether, the results obtained from the analysis performed immediately after exposure revealed a strong association between cytogenetic and DNA damage and proved that very low doses of X-rays like those assayed in this study are capable of inducing genotoxicity.

In addition, significant increases of micronuclei frequencies and index damage were obtained at delayed times in both cell lines. Similar findings were reported for doses higher than that used in this work (27–29) and for similar doses in our previous study in which cytogenetic alterations were analyzed at 12 and 16 population doublings after exposure to 50 mSv (12). These results support the hypothesis of a process by which initial DNA damage becomes permanent or is memorized by the surviving cells. In this sense, the results concur with SUZUKI et al. (43, 44) and LITTLE (45), who consider it unlikely that the 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. The same phenomenon was suggested in human stem cells by CHANG et al. (46),

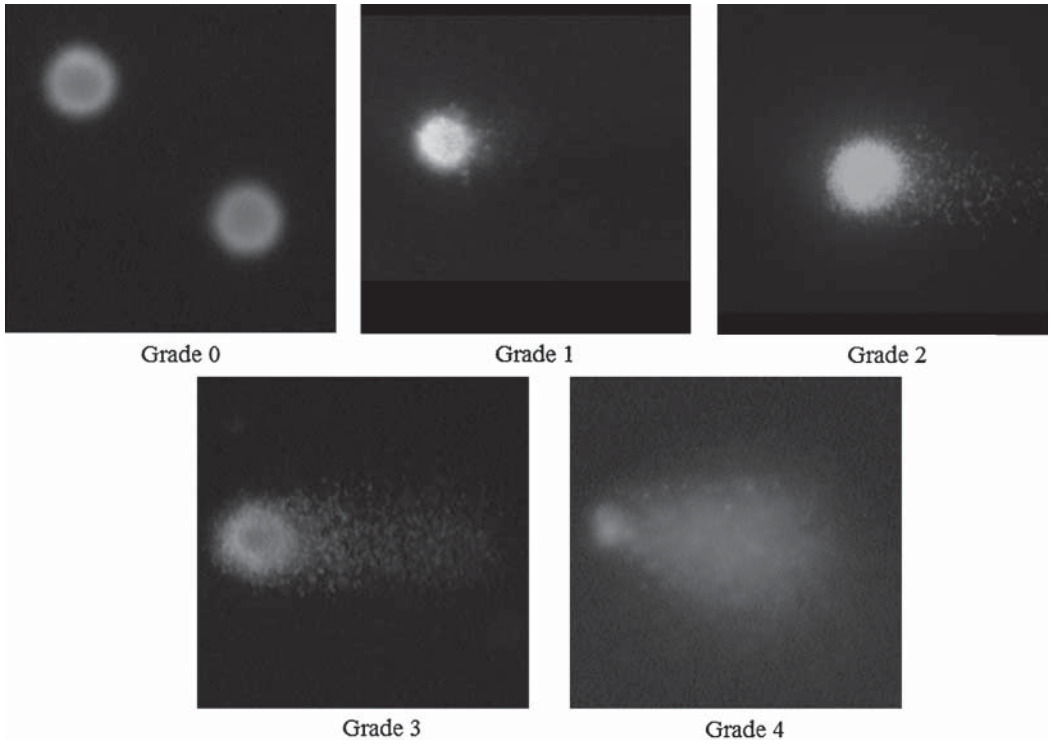

Fig. 1. Cell classification according to tail length. Categories: grade 0 (no visible tail) to grade 4 (still a detectable head of the comet but most of the DNA in the tail).

who found a persistent increase in the total micronuclei frequencies in exposed individuals relocated from radioactive buildings. Other authors also found genomic instability induction in cells of human origin (47–49).

On the other hand, comparative investigations between comet and micronucleus assays using chemicals and mutagens were carried out and the results showed that the micronucleus test seemed to be less sensitive to assess DNA damaging potential (17, 18, 23). The different sensitivities between techniques can be explained by taking into account that while chromosomal aberration analysis only detects the misrepaired DNA lesions persisting in the cell, the comet assay is intended to detect lesions in individual cells at an early stage after exposure, allowing a more efficient evaluation of the damage (33). Also, the ID appears to be a sensitive parameter for DNA damage evaluation as reported by other authors in different cell types (50, 51). Its use as a parameter of the comet assay was cited by MALUF et al. (22) and MARTINO-ROTH et al. (20) in human lymphocytes exposed to radiation doses similar to those employed in the present study. In summary, although comet and micronucleus assays are sensitive enough to assess this damage, we are providing additional evidence of the fact that the comet assay is more sensitive than the micronucleus test in the two cell types tested.

In conclusion, cytomolecular and cytogenetic damage can be induced in cells exposed to very low doses of ionizing radiation and their progeny. Although the mechanisms underlying ionizing radiation are not totally understood, our results show that low doses of radiation are sufficient to induce genomic instability. Taking into account that genomic instability may play a significant role in tumorigenesis (45, 47, 52), these findings have important implications for risk estimation associated with low-dose radiation exposure. Nevertheless, the results are limited to the conditions and cell type used. Further studies will be required to determine whether these effects are observed in different cell models and conditions, especially in cells of human origin.

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