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Reduced DNA damage in tumor spheroids compared to monolayer cultures exposed to ionizing radiation

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Background: Several cell lines when cultured under proper condition can form three dimensional structures called multicellular tumor spheroids. Tumor spheroids are valuable in vitro models for studying physical and biological behavior of real tumors. A number of previous studies using a variety of techniques have shown no relationship between radiosensitivity and DNA strand breaks in monolayer and spheroid model of cell culture. ***Materials and Methods:*** In the present study, the radiosensitivity of cells grown as monolayer and spheroid were measured with colony assay and the role of DNA strand breaks in this sensitivity was examined using single cell gel electrophoresis assay also known as Comet assay. ***Results:*** In the present experiment, spheroids showed more radioresistance than monolayers as judged by the number of colonies which they produced after radiation. Under the same experimental conditions, less level of DNA damage was detected in spheroids using "comet assay" technique. ***Conclusion:*** It was concluded that the loss of radioresistance which was observed in monolayer cultures might have been attributed to the higher level of DNA damage occurred in the cells. **Iran. J. Radiat. Res., 2007; 5 (2): 63-69**

Keywords: *Multicellular tumor spheroid, DU 145, radiosensitivity, DNA damage, comet assay.*

INTRODUCTION

Multicellular tumor spheroids (MCTS) are a well-established 3-D *in vitro* model system that reflects the pathophysiological *in vivo* situation in tumor systems. When grown in spheroids, cancer cells exhibit a phenomenon known as 'multicellular resistance' (MCR) [1].

Spheroids show more resistance to ionizing radiation as compared to monolayer cultures [2, 3]. This resistance appears to be due to the greater capacity of cells grown in contact to repair radiation damage. Attempts to relate this "contact effect" to differences in DNA susceptibility, or DNA repair capacity have provided conflicting results. The two techniques alkaline sucrose gradient sedimentation and alkaline elution, showed no difference in the amounts of radiation-induced DNA, single-strand breakage, or its repair between suspension or monolayer cells. However, using the alkali-unwinding assay, the rate of DNA unwinding was much slower for suspension cells than for monolayer cells. A fourth assay, sedimentation of nucleoids on neutral sucrose gradients, also showed a significant decrease in radiation damage produced in suspension compared to monolayer cultures. It is believed that this assay measures differences in DNA conformation (supercoiling) as well as differences in DNA strand breakage. By these four assays, it can be concluded that the same number of DNA strand breaks/Gy is produced in monolayer and spheroid cells. Of course changes in DNA conformation or packaging occur when cells are grown as spheroids, and these changes are responsible for reducing DNA damage by ionizing radiation[4]. The supercoiled structure of chromatin, as salt-extracted nucleoids, is examined using flow cytometry. Irradiated viable cells from spheroid culture contain restraints to supercoil relaxation that are absent in monolayer cells. Further

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analysis of the chromatin organization from each growth form shows that the radioresistant spheroid cells contain a DNA-protein matrix which is more resistant to detergent-induced degradation. It was suggested that the increase in structural integrity of the spheroid cells could explain their greater radioresistance by providing a more stable platform for high-fidelity DNA damage repair[5]. In another experiment although identical amounts of damage were produced in monolayers and spheroids by the topoisomerase I inhibitor camptothecin, and the cell cycle specific agent, 5-fluorouracil fewer strand breaks were induced by etoposide in spheroid DNA than monolayer DNA, as measured by the DNA precipitation and alkali unwinding assays. It was suggested that the decrease in the activity of this enzyme could be linked to the change in DNA conformation in spheroids and the decrease in their radiation sensitivity[6]. Another experiment suggested that the reduced radiosensitivity of V79 spheroid cells could not have been related to a reduced number of initial DNA lesions or a higher capacity to rejoin DNA breaks (measured by neutral elution). These findings suggested that the ratio of lesion repair to fixation/misrepair may differ between cells from spheroids and monolayer culture, thus influencing the cells' response to dose-rate changes differentially[7].

Comet assay was introduced in 1984 as a single-cell gel electrophoresis method[8] and later developed by others[9] is a simple, effective and quantitative method which allows evaluation of strand breaks in DNA at the level of single cells in neutral or alkaline pH. Good correlation between cell killing and DNA damage, measured by using alkaline comet assay, was demonstrated for several drugs. It was proposed that the DNA damage measured by using the comet assay to be an effective and quantitative method of predicting drug cytotoxicity in complex multicellular systems[10].

In this work we have used the alkaline comet assay to study the radiosensitivity of multicellular spheroid cells to determine: 1- If the DNA damage differs in the spheroids as compared to monolayers, and 2- If the DNA damage assayed by the alkaline comet assay can be used as quantitative method for prediction of radiosensitivity of tumor cells.

MATERIALS AND METHODS

Cell line

Human prostate carcinoma cell line DU 145 was obtained from ATCC (American Type Culture Collection, Manassas, VA) and maintained in RPMI 1640 (Gibco) supplemented with 10% fetal calf serum (FCS) (Gibco), 500U/mL of penicillin (Sigma) and 500mg/L of streptomycin (Jaberebn-Hayan).

Monolayer culture

Cells were cultured as monolayer, at a density of 10^4 cells/cm^2 in T-25 tissue culture flask (Nunc). Cultures were maintained at 37°C in a humidified atmosphere of 7.5% CO_2. Cultures were propagated or cells were harvested by trypsinizing cultures with 1 mM EDTA/0.25% Trypsin (w/v) in phosphate-buffered saline (PBS).

Spheroid culture

Spheroids were initiated using the liquid overlay technique [11] 5×10^5 cells were seeded in 100mm Petri dishes (Nunc) coated with a thin layer of 1% agar (Bacto agar, Difco, Detroit, MI, USA) with 10 mL of RPMI 1640 supplemented with 10% FCS. The plates were incubated at 37 °C in a humidified atmosphere of 7.5% CO_2. When microspheroids reach the diameter of 50-60 µm, they were transferred to spinner flasks (Techne) and allowed to spin at the velocity of 40 rpm until they reached to the diameter of 110-120 µm.

Irradiation procedure

Single cells from spheroid or monolayer culture were irradiated with Gamma radiation from a ^{60}Co source (Theratron 780, AECL, Canada) with a dose rate of 1.32 Gy/min. Cells from both cultures were exposed to gamma rays at 1, 3 or 5 Gy. The fourth sample was not irradiated (0 Gy).

Trypan blue exclusion assay

A suspension of irradiated and control single cells from either monolayer or spheroid cultures were mixed with trypan blue at ratio of 9:1. After a few minutes the mixture was examined under a light microscope (Leica, DMLS), and the blue cells were considered dead. The percentage of unstained cells to total number of cells was reported as viability for each category of cells.

Clonogenic assay

Irradiated and control single cell suspensions from either monolayer or spheroid cultures were seeded in 60mm Petri dishes (Nunc) and grown in RPMI 1640 supplemented with 10% FCS. The cells were incubated at 37°C in a humidified atmosphere of 7.5% CO_2 for two weeks. After this period, the colonies which contained a minimum of 50 cells were counted using an inverted phase microscope (Zeiss, Axiovert 405M) and the plating efficiency was determined.

Survival curve

Survival curves were generated by plotting the ratio of plating efficiency of samples at a given dose to the plating efficiency of controls versus radiation doses on a semi-log scale. These curves were fit to the equation:

$$S = 1 - (1 - e^{-D/D_0})^n \qquad (1)$$

and its first order approximation:

$$S = ne^{-D/D_0} \qquad (2)$$

by the least square regression to determine the n, D_0 and then D_q using the relationship:

$$D_q = D_0 \mathrm{Ln}\, n \qquad (3)$$

Where n is the extrapolation number, D_0 is the final slope of the curve and D_q is quasi-threshold dose.

Alkaline comet assay

The alkaline comet assay in this work was a modification of the method described by Singh *et al.* [9]. Ordinary microscope slides were coated with 1% normal melting point agarose. Approximately 10,000 cells were suspended in 100 µL of 0.5% low melting point agarose. The cell suspension was rapidly pipetted onto the first agarose layer. The slides were allowed to solidify, then immersed in freshly prepared lysis buffer (2.5 M NaCl, 100 mM EDTA, 10 mM Tris-base with 1% Triton X-100, pH=10) and incubated for an hour. From that point on, all the steps were performed at 4°C. The slides were removed from the lysis buffer and placed in a horizontal gel electrophoresis tank which was filled with fresh cold denaturation buffer (300 mM NaOH, 1mM EDTA, pH=13). The slides were left in the solution for 30 min. Electrophoresis was conducted in the same denaturation buffer for 30 min using 1V/cm voltage and a current of 300 mA. Following electrophoresis, the slides were washed in Tris buffer (0.4 M Tris-HCl, pH=7.5) to neutralize the excess alkali. Finally, the slides were stained with ethidium bromide (20 µg/mL). The individual cells or comets were viewed and photographed using a fluorescent microscope (Zeiss, Axioskop 2 plus) equipped with a CDD camera (Hitachi, KP-D20BP) and the photographs were analyzed by Comet Score® software. DNA damages were quantified as an increase in tail moment, the product of the amount of DNA (fluorescence) in the tail and the distance between the means of the head and tail fluorescence distributions.

Statistical analysis

Each experiment was set up in triplicate. Data were expressed as Mean ± standard error of mean and were analyzed by Student's *t-test*. A value of $P \leq 0.05$ was considered statistically significant.

RESULTS

Viability assay

Irradiation of cells either in monolayer or spheroid cultures did not change the viability of cells. Figure 1 shows the fraction of viable cells after several doses of ^{60}Co gamma radiation.

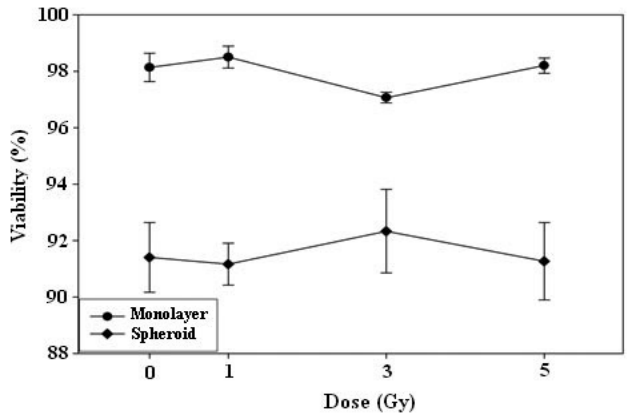

Figure 1. The effect of radiation on viability of DU 145 cells in monolayer and spheroid cultures. Immediately after radiation, viability of the cells was assayed using trypan blue dye exclusion test as described in the method section. Means ± SEM of three experiments.

Clonogenic assay

Irradiation reduced the clonogenic capacity of cells in a dose and culture dependent manner. Cells in spheroid culture showed more resistance to irradiation as compared to those cells cultured in monolayer. The cell-survival curves are shown in figure 2. Each curve shows an initial phase characterized by a positive shoulder followed by an exponential phase. To calculate the parameters of curves they were fitted to equation 2. The parameters for each curve are shown in table 1. Cells in the monolayer culture were twice as much sensitive to radiation than cells in spheroids, as judged by the slope of the exponential phase of the survival curves. However, both cultures showed similar extrapolation numbers.

Table 1. Parameters of radiation dose-response curve.

	Monolayer	Spheroid
D0	2.629776* ± 0.063155	5.467408 ± 0.129383
n	1.034581 ± 0.010169	1.044677 ± 0.005746
Dq	0.090279 ± 0.028362	0.239599 ± 0.033062

*Numbers are the mean±standard error of three independent experiments.

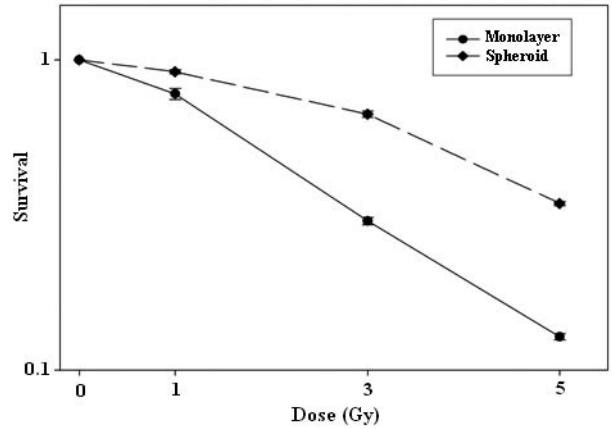

Figure 2. Radiation dose-response curve for DU 145 monolayer (circles) and spheroid (squares) cells. Different doses of radiation were applied to monolayer and spheroid cultures. Cells were then assayed for colony formation. The colonies formed 9 days after initiation of cultures were counted. Means ± SEM of three experiments.

DNA damages

The average of tail moments in each category of cells was used as an indication of DNA damage. In monolayer and spheroids, DNA damages increased as along with the increase of the radiation dose. The increase in DNA damage in monolayer cells was significantly greater than that of spheroids (figure 3).

The behavior of survival fraction and DNA damage after irradiation for both models of culture are illustrated in figure 4. To determine the correlation between survival and DNA damage, survival fraction of spheroid and monolayer cells were plotted versus tail moment of those cells. As seen in figure 5 the average tail moment has shown to be proportional to DNA damage present in

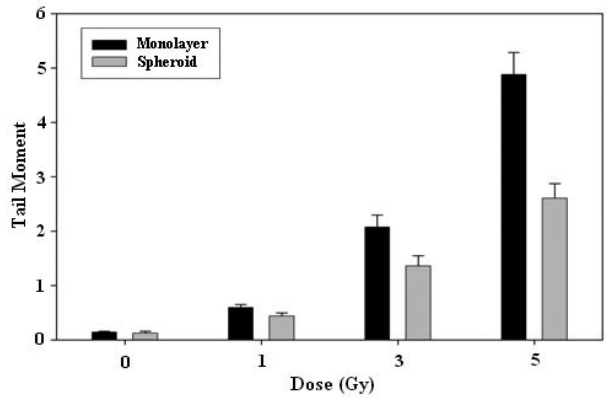

Figure 3. The effect of radiation on DNA strand breaks of DU 145 cells from monolayer and spheroid culture. Cells were exposed to different doses of radiation. Single cells were analyzed for DNA single strand breaks. Tail moment, an indication of DNA strand breakage was measured using the alkaline comet assay. Means ± SEM of three experiments.

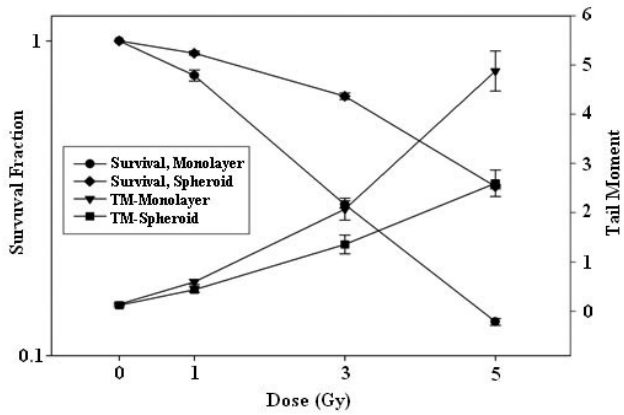

Figure 4. Cell killing and DNA damage by radiation in DU 145 cells from monolayer and spheroid cultures. Data extracted from Figure 2 and 3.

the cell [12] also, has shown correlation with the surviving fraction in DU 145 monolayer and spheroids, irrespective of the model of the culture. Correlation coefficient calculated for observed tail moments and survival fractions was 0.92 and 0.99 for monolayer and spheroid cultures respectively.

DISCUSSION

Special architectural characteristics of multicellular tumor spheroids have made it a useful model to study the effects of chemical drugs [10,13-15], hyperthermia [16-18], and radiation [19-21]. The aim of current study was to investigate DNA damage assayed by alkaline comet assay as a quantitative method for prediction of radiosensitivity of cells in both spheroid and monolayer cultures.

As shown in figure 4, the level of DNA strand breaks in DU 145 non-irradiated spheroid cells has been the same as those grown as monolayers. This indicated that spheroid formation was not a stress in inducing DNA strand breaks.

Figure 2 supports the hypothesis that monolayer cell cultures due to some structural and physiological differences with *in vivo* tumors might have lost their protective systems against environmental stresses such as ionizing radiation[22]. Figure 2 showed that ^{60}Co gamma radiation has reduced the clonogenic ability of both monolayer and spheroid cells in a dose and culture dependent manner. As it is concluded from figure 1, this reduction has not been due to the change in viability of cells in these two models of culture. However, the extent of reduction in number of clonogenic cells from monolayer cultures was significantly larger than those from spheroid cultures. Based on these results, it can be claimed that DU 145 cells acquire increased radioresistance when growing as multicellular spheroids. Similar results have been reported by other investigators using other cell lines [23] as well as some previously obtained similar data with this cell line using hyperthermia treatment[18].

Figure 4 depicts that in both monolayer and spheroids, the tail moment (the product of tail length and the amounts of DNA in the tail region) increases along with increase of the radiation dose, but the extent of the increase in monolayer cells has shown to be greater than that of spheroids; furthermore, the results in figure 5 and the correlation coefficients which were calculated showed that the average DNA damage in a population of cells to be a good indicator of cell killing especially in homogeneous system just like microspheroids with diameter of about 100 μm.

The way in which DNA is organized within the cell is known to influence its sensitivity to a variety of DNA damaging agents[24]. As an example it has been reported that the induction of strand breaks by radiation is generally greater in transcriptionally-active DNA [25] and changes in DNA structure through the cell cycle are accompanied by changes in radiosensitivity[26]. Previous studies have revealed that chromatin conformation in spheroid cells is different from that of monolayer cells[5, 27, 28]. We have concluded that the increased level of DNA damage observed in monolayer cells (figure 4), might be attributed to the particular architecture of their chromatin.

In conclusion, the result presented here has indicated that spheroid formation was not a stress-generating process in induction of DNA damage as compared to monolayer

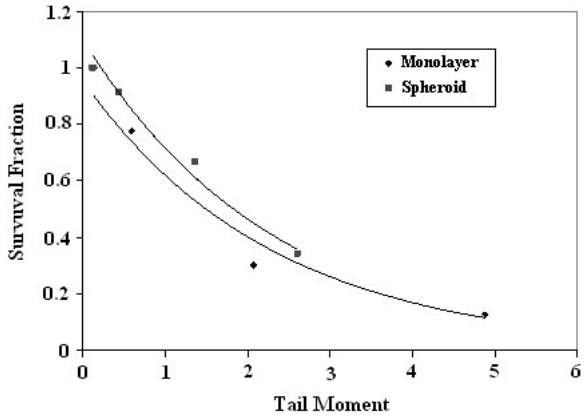

Figure 5. Correlation between tail moment, a measure of DNA strand breaks and cell killing by radiation for two models of monolayer and spheroid cultures. Cells exposed to radiation were analyzed for both DNA damage and cell killing.

culture of cells. Cells in the spheroid cultures showed increased resistance to ionizing radiation at all doses of radiation. Under the same experimental conditions, spheroids represented less level of DNA damage in comparison with monolayer cultures. Therefore the acquired radioresistance of spheroids may be attributed to the reduced level of DNA damage which itself is due to different chromatin packaging that is inherent in this model of culture.

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