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The role of Rad51 protein in radioresistance of spheroid model of DU145 prostate carcinoma cell line

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Background: Rad51 is a protein with critical role in double strand break repair. Down-regulation of this protein has a significant effect in radiosensitivity of some cell lines like prostate carcinoma. Compared to monolayer cell culture model, the spheroids are more resistant to radiation. The aim of the current study was to determine the Rad51 protein level in DU145 spheroids, and monolayer cells before and after exposure to gamma irradiation. ***Materials and Methods:*** In the present study, western blot was used to determine the level of Rad51 protein in DU145 cell line grown as monolayer and spheroid. ***Results:*** Western blot analysis showed that in the spheroid cells, Rad51 had an elevated level before and after radiation in comparison with monolayer cells. Higher doses of radiation induced elevated expression of Rad51 protein in both culture models. The level of at protein after exposure to gamma rays had been time-dependent. ***Conclusion:*** Rad51 might act as a mediator of radiation resistance in tumor cells. Repression of Rad51 activity could be a prominent strategy to overcome radiation resistance of tumors.
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INTRODUCTION

Multicellular tumor spheroids (MCTS) are intermediates of monolayer cell culture and *in-vivo* tumors. In the 1940s and 1950s, J. Holtfreter and A. Moscona, created the first *in vitro* method for cell aggregate and later in 1971, Sutherland and coworkers used this method in cancer studies [(1)]. Compared to monolayer cultures, the micro-environment of multicellular tumor spheroids shows more similarity to real tumors. The patterns of nutrition gaining and gas penetration are very similar in spheroids and cancer tumors. In spheroid model, the cell and its nucleus are round in shape, while in monolayer cells are disk-like [(2-3)]. In 1972 Durand and coworkers illustrated that spheroids of Chinese hamster V79-1716 cells, when exposed to ionizing radiation, were more resistant than cells grown as monolayer [(4)]. The difference in radiosensitivity was attributed to the cellular capability to repair DNA lesions [(5)]. Hinz in 1983 and Olive in 1985 showed that equal doses of radiation caused more radiation-induced mutations in monolayer cells than spheroids [(6, 7)]. The mechanism of difference in radiation resistance of spheroids and monolayer cells is generally called "contact effect". Contact effect can be due to a change in the cell shape and/or in the pattern of gene expression and protein level [(2)]. Therefore, it could be hypothesized that in spheroid cells a change in the mechanism of DNA repair might take place in comparison with cells in monolayer culture [(8)].

Rad51 protein is one of the key proteins involved in homologous recombination and DNA double strand break repair [(9)]. Rad51 proteins and bind the single strand DNA in double strand break points and form a nucleoprotein filament. This nucleoprotein filament migrates and finds the homologous

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sequence then bind it. Elevated level of Rad51 enhances radiation resistance in mammalian cells [10]. Rad51 down regulation using its specific anti-sense oligonucleotide leads to radiosensitivity of tumor cells in monolayer culture [11]. Down regulation of this gene in LNcap cells with an anti-Rad51 ribozyme was shown to have the same effect [12]. The goal of present study has been to measure the Rad51 protein level in spheroid and monolayer cells after exposure to gamma irradiation.

MATERIALS AND METHODS

Cell line

Human prostate carcinoma cell line DU145 was maintained in RPMI 1640 (Gibco) supplemented with 10% fetal bovine serum (FBS) (obtained locally), 500 IU/ml of penicillin (Sigma) and 200 mg/l of streptomycin (Jaberebn-Hayan, Iran).

Monolayer culture

Cells were cultured as monolayer in T-25 tissue culture flasks (Nunc) at the density of 10^4 cells/cm^2. Cultures were maintained at 37°C in a humidified atmosphere of 7.5% CO_2. 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 [13]. 5×10^5 cells were seeded into 100 mm Petri dishes covered with a thin layer of 1% agar (Bacto Agar, Difco) with 10 ml of RPMI-1640 supplemented with 10% FBS. The plates were incubated at 37°C in a humidified atmosphere of 7.5% CO_2. Half of the culture medium was replaced with fresh medium, twice a week.

Irradiation of monolayer cell cultures

Cells were cultured at 25×10^4 cells per flask in T-25 culture flasks, in RPMI 1640 culture medium supplemented with 10% FBS. To homogenize the rays reaching the cells, the culture medium of each flask was increased after 4 days to 0.5 cm to cover the cellular layer. The cells were exposed to 5 and 10 Gy of gamma irradiation with a Co-60 source, and a dose rate of 1.06 Gy/min. RADCAL software was used to calculate the time of irradiation. Control cells were not exposed to gamma irradiation. Irradiated cells were incubated at 37 °C in a humidified atmosphere of 7.5% CO_2, and then were harvested at different times after incubation for measurement of Rad51 protein level.

Irradiation of spheroid cultures

To study the effect of gamma irradiation on spheroids, the cells were cultured at 5×10^5 cells per Petri dish in 100 mm dishes and covered with a thin layer of 1% agar to elicit multicellular spheroid formation. After 13 days, the spheroids were gently precipitated and divided into T25 flasks. In order to homogenize the rays reaching the cells, the culture medium of each flask was increased to 0.5 cm to cover the cellular layer. The cells were then exposed to 5 and 10 Gy of gamma irradiation with a source of Co-60 with a dose rate of 1.06 Gy/min. Calculation of irradiation time was carried out using RADCAL software. Control cells were not exposed to gamma irradiation. Diameter of spheroids was 100µm in average. In that diameter it was expected to observe no hypoxia condition [23, 24]. The spheroids were incubated at 37°C in a humidified atmosphere of 7.5% CO_2, and then were collected at different times after incubation. Rad51 protein level was measured in the samples.

Protein determination

The cells collected from two 100 mm Petri dishes were washed with PBS and lysed at 4°C for 30 min in 200–300 µl of lysis buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.02% sodium azide, 0.1% SDS, 100 µg/ml PMSF , 1% (V/V) Nonidet P-40). The lysate was then centrifuged at 12,000 rpm for 20 min to pellet large cellular debris. Protein concentrations were measured using the method introduced by

Bradford [14].

Gel electrophoresis and Western blotting

Gel electrophoresis was performed in a 12% SDS polyacrylamide gel according to Laemmli [15]. Samples containing equal amounts of protein (150 µg) were mixed with 1/5 volume of sodium dodecyl sulfate polyacrylamide gel electrophoresis loading buffer (6x), then heated at 90 °C for 8 min, and were subjected to electrophoresis at 4 ° C, 200 V for 4 h. To perform the Western blot analysis, the separated proteins on the SDS gel were electroblotted to nitrocellulose (Schleicher and Schuell) and then probed with monoclonal anti-Rad51 (Gene Tex) and monoclonal anti-ß-actin (Sigma). ß-actin bands were detected to control loading. The primary antibody was detected with secondary anti-rabbit IgG conjugated with horseradish peroxidase to generate an enhanced chemiluminescent (ECL) substrate signal (Pierce). The antibody-labeled Rad51 polypeptide bands on the developed blots were digitally captured with a UVI Tec and band intensities were measured using Totallab® software.

Statistical analysis

Data were given as mean values ± S.D, and were calculated using the following equations in an error propagation procedure where "σ" is the Standard Deviation (SD).

(1) $U = x/y$ Rad51 = x and ß-actin = y

(2) $(\sigma U/U)\ 2 = (\sigma x/x)2 + (\sigma y/y)\ 2$

(3) $\sigma U = (x/y) \times ((\sigma x/x)^2 + (\sigma y/y)^2)^{0.5}$

RESULTS AND DISCUSSION

Cell characteristics

The DU145 prostate carcinoma cell line grew as a monolayer on plastic culture flasks with a population doubling time of approximately 33 h. The DU145 cells could form spheroids in liquid overlay cultures.

Effect of gamma radiation on spheroid and monolayer Rad51 protein levels

The levels of Rad51 protein were measured at 45 min. 12 and 24 hours after exposure to gamma radiation. Figure 1 shows the western blot detection of Rad51 protein from control and irradiated monolayer and spheroid cells. The results of western blot analysis showed that when monolayer cells were exposed to 5 Gy of gamma radiation, the level of Rad51 protein slightly had increased after 12 h (figure 2A). After 24 h, however, the level of Rad51 protein showed 43% increase compared to the control. In spheroids exposed to the same dose of irradiation, after 45 minute and 12 h, the level of Rad51 protein increased to 38% and 59.5%, respectively, but it was decreased, after 24 h (34.34% increase compared to control). These results, in general, showed that after 5 Gy of irradiation, an increase in Rad51 protein level would occur faster and more significantly in spheroids in comparison with monolayer. The process was followed by a decrease, after 24 hours post-irradiation (figure 2A).

The changes in Rad51 protein level in spheroid and monolayer cells after 10 Gy of gamma irradiation are depicted in figures 1 and 2B. As shown in figure 2B, an increase in Rad51 protein level has been observed 12 h after treatment of monolayer cells with 10 Gy of gamma radiation. In these cells, comparing to non irradiated control cells, there was a slight increase (24%) in Rad51 level, 12 hours after 10 Gy of irradiation and a greater increase (58.5%) in the protein level was observed 24 hours postir-radiation. Interestingly, 45 min and 12 hours after 10 Gy of gamma irradiation, the level of Rad51 protein in spheroid cells increased significantly, to over 91 and 74% of the control level, respectively. 45 min and 12 hours after 10 Gy of gamma radiation treatment, the increase in Rad51 protein was more conspicuous in spheroid cells than in monolayer cells; however, 24 hours after 10 Gy of gamma irradiation, a decrease in the level of the protein was observed in spheroid cells in comparison with monolayer.

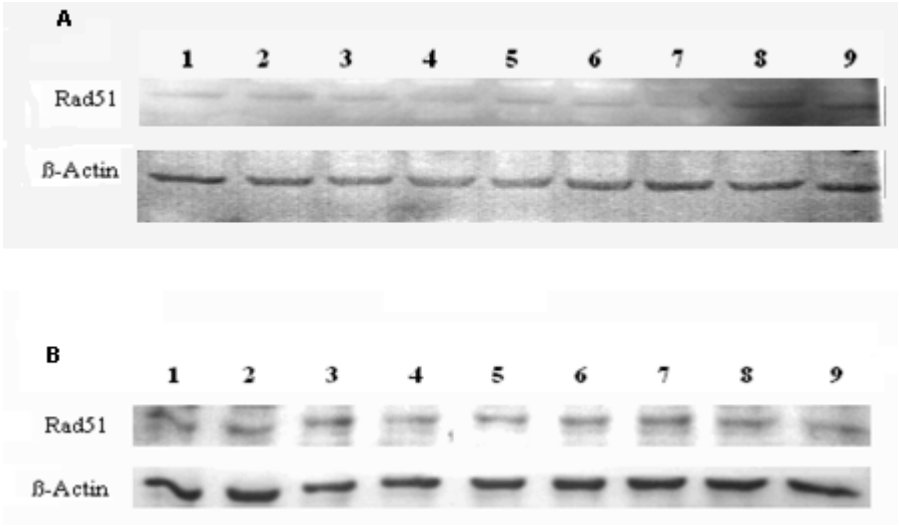


Figure 1. Western blots of Rad51 protein from control and gamma irradiated monolayer (A) and spheroid cells (B). Lanes 1-3: Rad51 protein level 45 min after 0, 5 and 10 Gy of IR treatment, respectively; Lanes 4-6: Rad51 protein level 12 h after 0, 5 and 10 Gy of IR treatment, respectively; Lanes 7-9: Rad51 protein level 24 h after 0, 5 and 10 Gy of IR treatment, respectively. ß-actin was used as loading control.

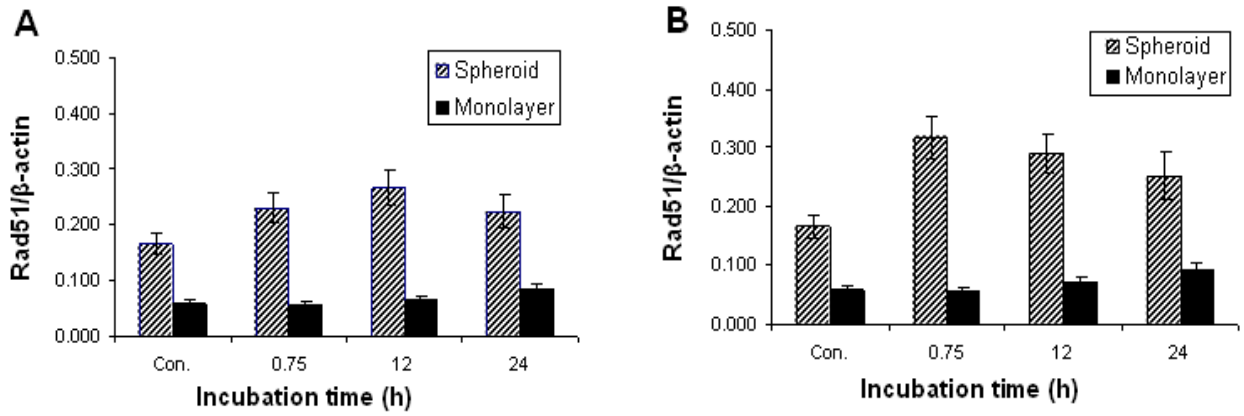


Figure 2. Rad51 protein level in spheroids and monolayer cells at different incubation times after gamma irradiation The protein level was expressed as a ratio of Rad51/β-actin A) Rad51 protein level of monolayer and spheroid cells was measured at various times of incubation after 5 Gy of gamma irradiation. B) The level of Rad51 in monolayer and spheroid cells at various times of incubation after 10 Gy of gamma irradiation.

Figure 3 demonstrates the level of Rad51 protein as a function of gamma irradiation dose. In monolayer cells, 45 min after radiation with 5 and 10 Gy of gamma rays, no significant change in the level of Rad51 protein was observed (figure 3A). In contrast, Rad51 level in spheroid cells showed an increase, especially at the higher dose (91.5% compared to control). 12 hours post 10 Gy of irradiation. There was a slight increase (24%) in monolayer cells protein level, however in spheroid cells, compared to non irradiated control cells, 59.5% and 75% increase in Rad51 was observed after 5 and 10 Gy of irradiation (figure 3B). A significant increase after 5 and 10 Gy of irradiation of monolayer cells, as well as spheroids, was detected 24 hours after irradiation (figure 3C). After 5 Gy of gamma irradiation, the Rad51 protein level in monolayer cells increased to 43% of the amount of control cells, and 58.5% increase was observed after 10 Gy of radiation. Similar to monolayer cells, Rad51 protein level in spheroids, also, showed 34% and 51% increase, 24 hours after 5 and 10 Gy of gamma irradiation, respectively. The results showed that the expression of Rad51 protein in spheroids and monolayer cells to be time- and dose-dependent.

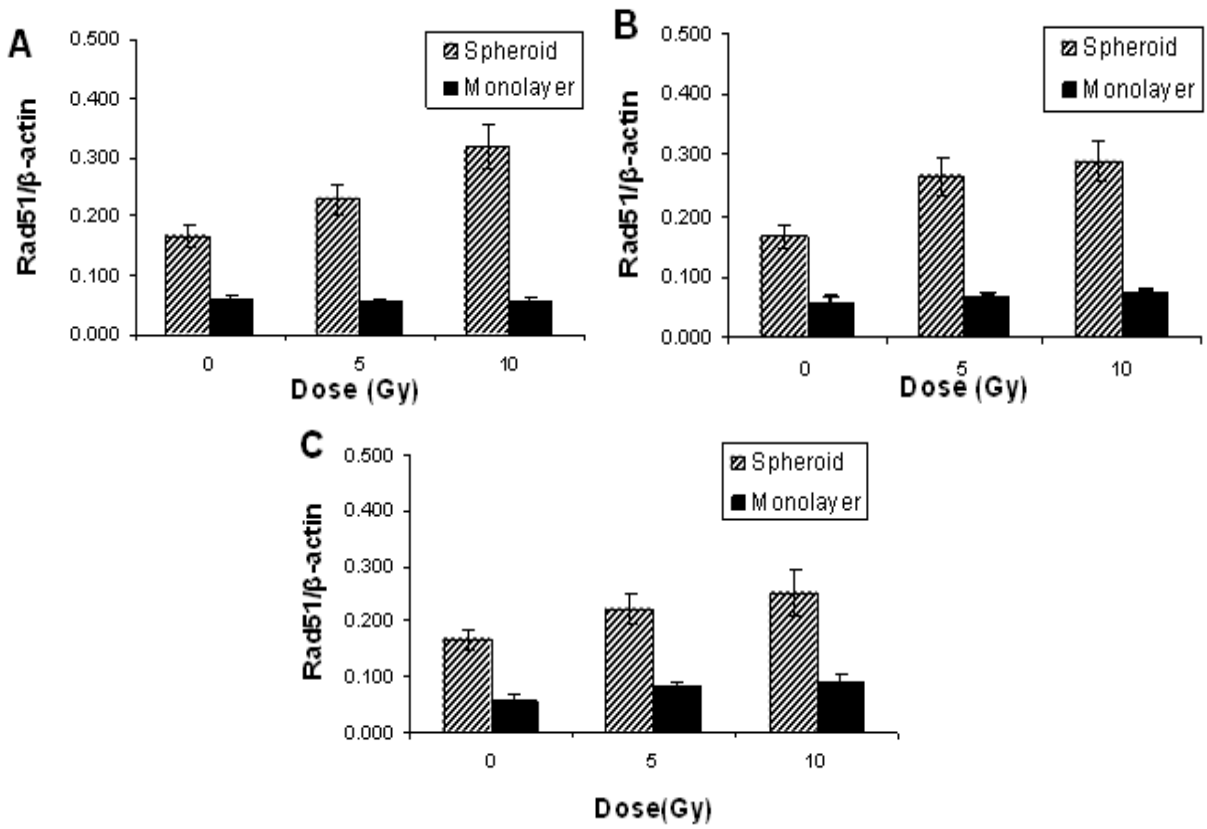


Figure 3. Rad51 protein level in spheroid and monolayer cells after irradiation with different doses of gamma rays. Each graph was depicted for various incubation times after irradiation. The protein level was expressed as a ratio of Rad51/β-actin. A) Rad51 protein level of monolayer and spheroid cells was measured 45 min (A), 12 (B) and 24 hours (C) after 5 and 10 Gy of gamma radiation.

CONCLUSION

Numerous attempts have been made to overcome the resistance of solid tumors against radiotherapy. Identification of different factors involved in radio-resistance and understanding their behavior against radiation could be a prominent step towards cancer radiotherapy. Molecular mechanisms that contribute to differences in radio-resistance include variations in DNA repair mechanisms. In mammalian cells, the product of the rad51 gene mediates DNA repair via homologous recombination. Rad51 overproducing cells were shown to be radio-resistant [10]. In contrast, Rad51 down regulation by a specific ribozyme or antisense inhibition increases radiosensitivity of tumor cells [11, 12]. Taken together these results suggest that there is a direct relationship between Rad51 protein level and radiation resistance of tumor cells. As Rad51 is an essential protein in DNA repair; measurement of its expression level in the cell can be a powerful tool to explore its function in tumor biology. In the present study, the researchers aimed to investigate the effect of radiation dose, time, cell contact and phenotype on Rad51 protein level. Western blot analysis showed that both before and after ionizing radiation, the level of Rad51 protein in DU145 cells was about three to five folds higher in spheroids than in monolayer cells. The result was in agreement with previous studies and indicated that in contrast to conventional monolayer cell systems, Rad51 protein accumulates high-levels in three-dimensional cell culture models [16]. On the other hand, in a study on V79 Chinese hamster cells, it was illustrated that the cells grown as spheroids were more resistant against gamma irradiation than those in monolayer culture [5]. In a previous study it was also observed that

spheroids of DU145 cell lines, in comparison with monolayer cells of this cell line, to be more resistant to gamma irradiation (Results not shown). These results reconfirm the role of Rad51 in resistance of tumor cells to ionizing radiation.

After exposure to ionizing radiation, spheroids expressed the Rad51 protein faster and more efficiently than monolayers. 45 minutes after 10 Gy irradiation, the Rad51 level increased significantly (100%) in spheroids, while in monolayers 24 h after application of the same dose of irradiation 60% increase was observed in the level of the protein. This can describe the reason why the radiation-induced G_2 block (which was believed as the time necessary for DNA repair before mitosis onset) is significantly shorter in spheroids than monolayers [17,18].

Previous studies showed that equal doses of radiation induce fewer mutations in spheroids than in monolayers [6,7]. For the same applied doses of radiation we applied, higher expression levels of Rad51 was observed in spheroids than in monolayer which is in agreement with the role of Rad51 in radiation-induced double strand break repair [19]. Moreover, when the cells were exposed to a higher dose of irradiation, higher levels of Rad51 were expressed. The increase was observed in both spheroid and monolayer cell culture models.

The results indicated that after exposure to ionizing radiation, the Rad51 protein level in the cells would be dependent on several factors including dose of irradiation, cellular shape and contact and also the time lapsed after irradiation.

There are reports indicating that the cell signaling processes can change as a result of cell shape and contact (spheroids versus monolayer). For example, in spheroid outer cells versus monolayer cells an increase in tyrosine phosphatases was reported [20]. These alterations, in turn, can affect DNA repair mechanisms [21] and cellular response to ionizing radiation. It is suggested that activation of C-Abl tyrosine kinase could causes an elevation in Rad51 protein level in lymphoma and leukemic cells [22]. But the mechanisms and signaling pathways which are involved in Rad51 mRNA induction and Rad51 protein stability in spheroid and monolayer cells after irradiation are not clear.

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