Assessment of Radiation-Induced Bystander Effect in Astrocyte-Glioblastoma Cell Lines

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ABSTRACT

In this study, the bystander effect on reactive oxygen derivatives (ROS) production and DNA damage level in normal astrocytic cells was examined using a co-culture method mimicking boundary conditions between normal astrocytes and cancer cells in the glioblastoma (GBM) tumor. SVG-p12 astrocytes and U87-MG GBM cells were co-cultured. ROS production and DNA damage were determined using flow cytometer after ionize radiation (IR) treatments of 2Gy and 4Gy doses. The one-way analysis of variance (ANOVA) was used to evaluate differences be-tween means of groups. Spearman's rank correlation coefficient was used for correlation analysis. We found that the percentages of Reactive Oxygen Species (ROS) productions were increased in all experimental control groups after 2Gy (U87-MG: 51.6%, SVG-p12: 34.3%, coSVG-p12: 19.1% and coU87-MG: 50.2%) and 4Gy (U87-MG: 41.2%, SVG-p12: 21.8%, coSVG-p12: 22.3% and coU87-MG: 26.5%) treatments. In addition, the increased radiation dose and pro-longed incubation period induced Double Strand Break (DSB) in the U87-MG cells co-cultured with astrocyte cells ($p< 0.05$). The transfer of medium irradiated with 4Gy dose increased ROS levels but not DSB in co-culture. Our study shows that RIBE arising from astrocyte cells in the irradiation area may induce ROS production and DSB in GBM cells. Cellular debris of radiation-disrupted astrocytes may cause RIBE altering response of GBM cells to IR.

Keywords: Astrocyte, Glioblastoma, Bystander effect

INTRODUCTION

The majority of primary brain tumors in the central nervous system are originated from glial cells.[1] The most common type of glial tumors is glioblastoma (GBM) tumors. GBM is the most aggressive glial tumor with poor prognosis.[2,3] The GBM cells are known to be drug-resistant 4 and highly invasive cells that can infiltrate into the surrounding normal brain tissue.[5] Thus, only surgical treatment for patients with GBM is often insufficient and the chemotherapy with the local radiation therapy is carried out following the surgery.[6,7] Although radiotherapy leads to apoptotic cell death in the malignant tumor cells due to DNA damage induced by ionizing radia-tion (IR), studies have indicated that IR may cause unwanted side effects on the normal cells in the treatment area.[8,9]

IR can damage DNA in the normal cells either directly by breaking the phosphodiester back-bone or indirectly by inducing the production of free radical species in the area been treated. However, normal cells which are not exposed to radiation at the near outside of the treatment area may receive the radiation effect by the irradiated cells. This phenomenon is the radiation-induced bystander effect (RIBE). Studies have demonstrated that RIBE can emerge from secret-ed factors by the irradiated cells and Gap junctions between cells.[8,10]

Molecular mechanisms of RIBE have been extensively studied in brain cancer cell models. RIBE in GBM cells have been demonstrated using various in vitro methods. Faqihi et al., used medium transfer technique to induce RIBE in GBM cells and found that the expression of pro-apoptotic JNK gene is upregulated and the expression of anti-apoptotic BCL2 is downregulated in GBM cells due to RIBE. They also suggested that activated ROS may increase JNK gene expression depending on RIBE. However, their study has only focused on differentially expressed apoptotic genes rather than evaluating ROS and DNA damage levels for RIBE.[11] Although RIBE has been demonstrated experimentally both in vitro and in vivo, only a few studies have addressed a possible DNA damage induced by ROS products in GBM cells in terms of RIBE.[12,13]

Here, we aim to evaluate the ROS and DNA damage levels in the irradiated astrocyte cells interacting with the irradiated GBM cells. For this purpose, we used the migration assay to co-culture GBM and normal astrocyte cells on the same surface, which would mimic cell-cell interactions in tumor tissue. We also test whether only irradiated media would have an effect on the ROS and DNA damage levels in the normal astrocyte cells.

MATERIALS AND METHODS

Cell Culture

Human GBM (U87-MG) and human astrocyte (SVG-P12) cell lines were obtained from Ege University Faculty of Medicine, Department of Histology and Embryology (Bornova, İZMİR). U87-MG and SVG-P12 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco) supplemented with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin, and 1% L-glutamine. The cells were incubated at 37^0C in the humidified atmosphere of 5% CO2 in the air.

Preparation of Agarose Gel

Two grams of agarose (Sigma, St. Louis, Missouri, USA) were added to 50 ml of phosphate salt buffer (PBS, pH: 7.14) for preparing 2% agarose gel. Circular agarose gel pieces with two opposite holes, about 2-3cm diameters, were prepared for co-culture. The prepared gel pieces were placed carefully in the wells of the six-well plates. The agarose gel pieces were sterilized by exposing them to ultraviolet (UV) light for 15 minutes.

Cultivation of Cell Groups in 6-Well Plates

GBM and astrocyte cells (1x104 cells in a well) were separately seeded into six-well plates. For co-culture, GBM and astrocyte cells ($1x10^2$) were seeded into the opposite holes on agarose gel in the well of the six-well plate. After 72 hours of incubation, these gels were gently removed from the wells. Three ml of culture medium were added to each well.

Irradiation of Cells

Irradiation of cells was applied using a specially designed solid water phantom for a six-well plate. The setup was scanned by CT (Toshiba Asteion, Japan) and the monitor units for IR doses were calculated by treatment planning system (XiO, Elekta, Sweden) using a 6MV (maximum dose depth (dmax)= 1.5 cm depth along the central axis) energy. Elekta Precise Linac (Elekta, Sweden) was used for IR with 300 MU/min accelerator mode and portal angle degree of 1800. 14 Doses of 2Gy-IR and 4Gy-IR were applied to each one of cell groups. After radiation treatment, cells were grown at 37^0C in a 5% CO2 atmosphere for 2, 4, 6, and 8 hours. After incubation, TUNNEL-based DNA fragmentation application and ROS measurements were performed.

Conditioned Cell Medium Transfer

To induce the bystander effect, the conditioned medium (CM) of the co-culture cell group irradiated by 4Gy radiation was transferred onto the non-irradiated co-culture cell groups. Cells in co-culture were incubated in CM for 8 hours.

The Detection of Cell Cycle Profiles

To identify ploidy status in characterization of U87-MG and SVG-p12 cells, BD cell cycle kit was used according to the manufacturer's instruc-

tions (BD cell cycle kit, BD Biosciences, Franklin Lakes, USA). Samples were read using the flow cytometry (BD Accuri C6 flow cytometry, Becton–Dickinson, USA). The fluorescence detector with the 585/40 bandpass filter was used to show the PI-DNA content (FL2- A) for the analysis.

In situ Direct DNA Fragmentation (TUNEL) Assay

Apo-BrdU in situ DNA fragmentation assay kit (BioVision, Inc. San Francisco) was used to perform in situ direct DNA fragmentation assay (TUNEL) according to the manufacturer's instructions. Cells ($5x10^6$ cells/ml) were harvested from six-well plates and fixed overnight at -20^0C in 70% (v/v) ethanol in ddH_2O. DNA double-strand break (DSB) mediated apoptosis was measured utilizing Br-UTP (bromolated deoxyuridine triphosphate nucleotides). DSB amounts in the cell line groups were compared with the positive and negative controls using flow cytometry at Ex / Em= 488 / 623 nm (PI).

Measuring Reactive Oxygen Species Level

Intracellular ROS levels were measured using the "ROS Detection Assay kit" (BioVision, Inc. San Francisco) according to the manufacturer's instructions. ROS amounts in the treated cell groups were compared to the positive control (1X ROS label supported by manufacturer). All cell groups treated with 1X ROS inducer were analyzed using the FL-1 bandpass filter of flow cytometer. ROS levels were measured at 6th hours of incubation following 2Gy and 4Gy radiation treatments.

Statistical Analysis

Statistical analysis was performed with the "SPSS statistics 17.0" programs. The one-way analysis of variance (ANOVA) was used to determine differences among percentage means for all treated groups. Spearman's rank correlation coefficient was used to show the correlation relationship between samples. p values of less than 0.05 considered statistically significant (the symbolic asterisks as $p< 0.05$*, $p< 0.01$**, $p< 0.001$***).

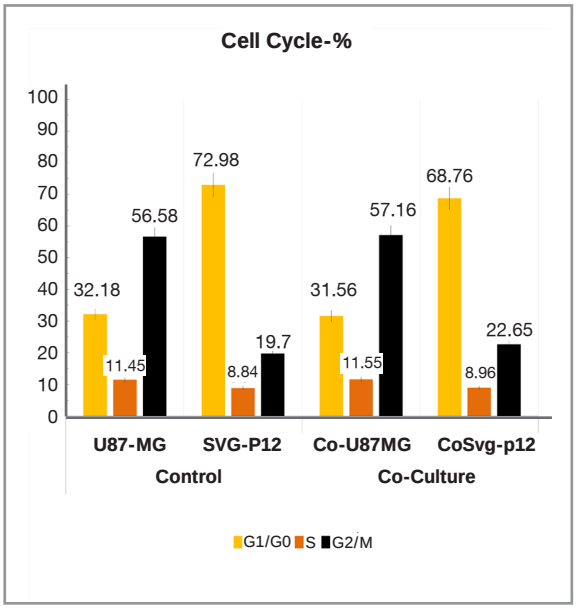

Figure 1. Percentage plots of cell cycle assay. Flow cytometry data of SVG-P12 and U87-MG cell lines (Control) and co-cultured (Co-SVG-P12 and Co-U87-MG) cell lines.

RESULTS

There is a border between two different cell groups in co-culture

GBM and astrocyte cells co-cultured on same well surface were characterized depending on the ploidy numbers to show whether there was a boundary between different types of cell in the co-culture. U87-MG cell line has aneuploidy karyotype and SVG-p12 cell line has diploid karyotype. 15 We found that GBM cells in both co-culture and sole culture had low tetraploid karyotype. In addition, SVG-P12 cells in these culture conditions had diploid karyotype (Figure 1). Our results indicated that two different cell types contacted each other on same well surface but not totally mixed.

The radiation treatment increases ROS levels in GBM and astrocyte cells

Dichlorofluorescein diacetate (H2DCFDA) probe was used to measure the effect of radiation on ROS production in GBM and astrocyte cells. ROS levels were determined after 6th-hour incubation following 4Gy and 2Gy IR treatments. Intracellular ROS percentages of cells exposed to IR were shown in Figure 2. Our findings demonstrated that

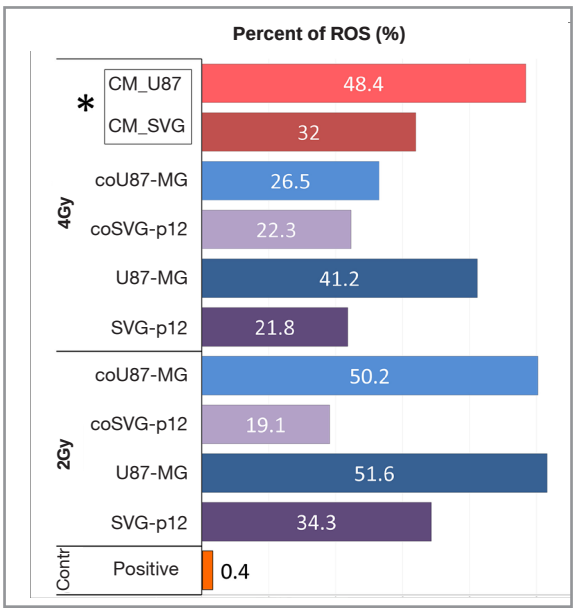

Figure 2. ROS detection by flow cytometry after 6 hours of incubation following IR. Groups of cells treated with 2Gy and 4Gy IR. * CM: Culture Medium.

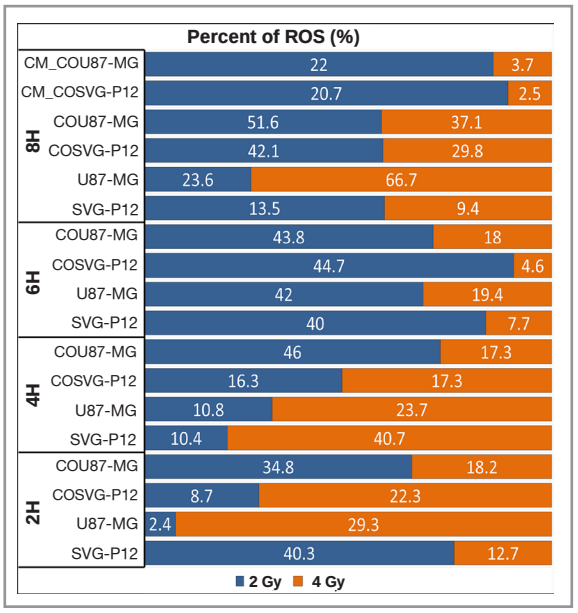

Figure 3. TUNNEL-based DNA fragmentation detection method. Flow cytometry data of in specified time intervals post-2Gy IR and post-4Gy IR. Percentage (%) data table for the DSB (Q1-UR) region for all cell groups.

direct radiation treatment increased the production levels of ROS in U87-MG and SVG-P12 cells in radiation-dose dependent manner when compared to positive control. In addition, the transfer of medium irradiated with 4Gy dose increased ROS levels in co-culture (Figure 2).

The radiation treatment indirectly increases DSB levels in GBM and astrocyte cells

The TUNEL-based DNA fragmentation detection method was used to determine indirect effect of IR on the genetic material. U87-MG and SVG-p12 cell lines were exposed to IR doses of 2Gy and 4Gy and they were incubated for 2, 4, 6, and 8 hours after radiation treatments. dsDNA percentages of U87-MG and SVG-p12 cells after radiation treatments are given in Figure 3.

We found a statistically significant difference in dsDNA break levels ($p < 0.05$) between co-cultured and non-co-cultured groups both of which were exposed to IR. In addition, the longer incubation periods were applied, the more dsDNA breaks were observed. On the other hand, irradiated culture media with 4Gy dose did not increase dsDNA breaks in co-culture. We also showed that the DSB-induced cell deaths were increased in cell groups (SVG-p12 and U87-MG) when IR dose was increased, and the incubation period was prolonged. When the co-cultured cell groups treated with 2Gy IR dose were compared according to incubation periods, we observed that the highest DSB amount was found for the 4hours incubation. For 2Gy radiation dose, whereas, SVG-p12 cells solely cultured and irradiated had lower DSB level than the U87-MG cells solely cultured.

The result of Spearman's correlation analysis in evaluating of the relationship between radiation doses and incubation periods for DSB-induced apoptosis is given in Table 1. In co-culture group treated with 2Gy radiation, we observed a high positive correlation between DSB and incubation periods. In addition, a high positive correlation was found in both co-cultured and non-co-cultured groups, all of which were treated with 4Gy radiation dose. We found that radiation dose was determinant factor for increased DSB in time-dependent manner. On the other side, co-culture group exposed to 4Gy dose had a higher correlation coefficient compared to co-culture group exposed to 2Gy dose.

Table 1. Spearman's rank correlation coefficient data

	2Gy-IR	2h	4h	6h	8h
SVG-P12	Correlation Coefficient	0.905**	0.857**	0.429	0.667
	Sig. (2-tailed)	0.002**	0.007**	0.289	0.071
	N	8	8	8	8
U87-MG	Correlation Coefficient	0.905**	0.405	0.167	0.524
	Sig. (2-tailed)	0.002**	0.320	0.693	0.183
	N	8	8	8	8
coSVG-P12	Correlation Coefficient	0.857**	0.244	0.905**	0.929**
	Sig. (2-tailed)	0.007**	0.560	0.002**	0.001***
	N	8	8	8	8
coU87-MG	Correlation Coefficient	0.762*	0.690	0.976**	0.905**
	Sig. (2-tailed)	0.028*	0.058	0.000***	0.002**
	N	8	8	8	8
	4Gy-IR	**2h**	**4h**	**6h**	**8h**
SVG-P12	Correlation Coefficient	0.881**	0.781*	0.946**	0.810*
	Sig. (2-tailed)	0.004**	0.022*	0.000***	0.015*
	N	8	8	8	8
U87-MG	Correlation Coefficient	0.310	0.738*	0.881**	0.286
	Sig. (2-tailed)	0.456	0.037*	0,004**	0,493
	N	8	8	8	8
coSVG-P12	Correlation Coefficient	0.287	0.810*	0.905**	0.667
	Sig. (2-tailed)	0.490	0.015*	0.002**	0.071
	N	8	8	8	8
coU87-MG	Correlation Coefficient	0.810*	0.929**	0.810*	0.857**
	Sig. (2-tailed)	0.015*	0.001***	0.015*	0.007**
	N	8	8	8	8

p< 0.05 *, p< 0.01 **, p< 0.001 *** significance value. Negative correlation (r < 0.20); low positive correlation (r= 0.20-0.39); medium positive correlation (r= 0.40-0.59); high positive correlation (r= 0.60-0.79) and very high positive correlation (r= 0.80-1.0).

DISCUSSION

Responses of GBM and astrocyte cells to radiation are quite different. In the current study, we evaluated the radiation-induced bystander effect (RIBE) on ROS and DSB levels in co-cultured normal astrocyte (SVG-P12) and GBM (U87-MG) cell lines. Studies have shown that glioblastoma is more resistant to ionizing radiation than normal brain cells.[16-19] However, we observed that U87-MG GBM cells were more sensitive to radiation. When GBM cells were cultured with astrocyte cells, the radioresistance of GBM cells were significantly increased.

Ionizing radiation can show various adverse effects on the non-transformed cells during treatment.[20-23] In addition, after radiotherapy, cells of the normal brain tissue can be affected by radiation exposed tumor cells in the treatment area. This phenomenon is called RIBE.[24-27] Typically, in tumor mass, glioma cells were surrounded by normal brain tissue cells and these cells are highly infiltrative. 5 Thus, astrocyte cells in the treatment area can be affected by IR. Astrocyte cells should not be examined separately from glioma cells for identifying RIBE. For this purpose, we used a co-culture method inspired by migration assay that can simulate high infiltration of normal cells into GBM cells. We found that

DSB-induced apoptosis and intracellular ROS levels in U87-MG cells co-cultured with SVG-P12 cells were higher than in U87-MG cells cultured alone after radiation treatments. Also, we observed that SVG-P12 cells cultured alone were more resistant to radiation treatment than U87-MG cells culture alone in terms of DSB induced apoptosis.

Although very little is known about RIBE, only few studies have indicated that RIBE is associated with increased invasion and metastasis activity in cancer cells. Thus, RIBE is also thought to be a reason of tumor recurrence after RT. The RIBE has been widely identified in different types of cancer.[28-33] In vitro experiment models have been successfully applied in these studies. The conditional media transfer has been widely used in co-culture studies to show indirect RIBE.[34-36]

So far, various effects of RIBE on non IR-treated cells have been reported.[34-36] The transferring of conditioned medium obtained from culture of irradiated GBM cells is an effective approach in showing of changes in biological processes of non-irradiated cells such as cell-survival, angiogenesis, invasion, and metastasis.[27] In this reason, we transferred the medium, which was used for co-culture and irradiated with 4Gy dose, into non-irradiated co-culture. Following this, ROS levels were increased but there were not significant difference in DSB levels. Consistently, Ivanov et al. did not observe RIBE effect on GBM cells using conditioned medium.[37] Faqihi et al. indicates that RIBE effect may not be detected in 2D in vitro culture conditions.[11] However, we detected twofold higher percentage of DSB-induced apoptotic cells in U87-MG cultured alone than in co-cultured GBM cells. Studies have shown interplay between DNA repair and cellular cytokines.[38-41] Thus, DNA repair-related cytokines secreted by SVG-P12 cells in the co-culture may decrease DSB levels in GBM cells after radiation treatment.

Another critical point in this study was that all cells in the well of six well-plates were ensured to receive full dose of IR. The beam qualities suitable for the IR were checked using a solid water phantom specially designed for six-well plates. 14 Penetration, homogeneity, and dose efficiency of beam could be kept.[42] DSB-associated apoptosis in co-cultured cells after IR treatments were evaluated by measuring the intracellular ROS products induced by RIBE. ROS production indicates indirect effect of RIBE, whereas changes in DSB levels indicate direct effect of RIBE. Studies have shown that astrocyte cells are resistant to oxidative stress and these cells protect other cell types from various stress conditions.[43-45] An increase for ROS level in the U87-MG cells in co-culture was observed at 6th hours of incubation after 2Gy treatment. At same condition, DSB-induced cell death was reduced. We found that the ROS levels and DSB mediated apoptosis were decreased after 6 hours of incubation for co-culture group when treated with 4Gy dose. We suggested that toleration of U87-MG GBM cells to ROS product may be result from the presence of SVG-P12 cells in the co-culture.

We observed that GBM cells cultured alone were radiosensitive for 2Gy and 4Gy doses. However, we demonstrated that GBM cells in co-culture were more resistant to radiation treatment. Thus, results indicate that RIBE may increase ROS levels and DSB-induced apoptosis in co-cultured GBM cells.

Conclusion

Our study shows that RIBE arising from astrocyte cells in the irradiation area may induce ROS production and DSB in GBM cells. Cellular debris of radiation-disrupted astrocytes may cause RIBE altering response of GBM cells to IR.

Acknowledgement:

Ege University Scientific Research Coordination supported this study (BAP, project no: TYL-2018-20105).

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