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Characterization of Radioresistant Variant From U251 Human Glioblastoma Cell Line and the Role of Antioxdant Enzymes in Its Radioresistancy

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Abstract

To investigate the radioresistant mechanism in glioblastoma multiforme(GBM), we isolated the radioresistant clone (RRC) from U251 human glioblastoma cell line by exposing to repeated fractions of 3 Gy γ-radiation for six months. RRC had higher radioresistance than the parent cell line as measured by clonogenic survival assay. FACS analysis showed that RRC had a delayed G2 arrest after radiation. Antioxidant enzymes, such as SOD, catalase, glutathione peroxidase (GPX), glutathione reductase (GR), were activated up to 5 folds in RRC after radiation. Erk 1/2 activation was higher in RRC than in the parent cell. Therefore, radioresistancy in RRC might be due to the delayed cell cycle, the coordinated high activation of antioxidant enzyme rather than a single enzyme alone,and higher activation of Erk 1/2.

1. Introduction

Malignant gliomas comprise more than 40% of central nervous system malignancies (1). Radiation therapy is used extensively in the primary management of patients with glioblastoma multiforme (GBM). GBM is one of the most resistant of human tumors to radiation whether used alone or in combination with surgery and/or chemotherapy. Despite

the use of radiation therapy and intensive clinical efforts to improve therapy, the median survival remains less than 1 year (2). This cellular intrinsic radiosensitivity may be a major determinant of the outcome of radiotherapy. One way to investigate how cells respond to ionizing radiation is to isolate mutant cell lines that differ from their parent lines in their radiosensitivity or radioresistancy.

Ionizing radiation has been known to enhance the production of reactive oxygen species(ROS) in a variety of cells. This intracellular formation of reactive oxygen species (ROS) is thought to be the major mechanism by which ionizing irradiation causes cell damage and death. ROS, mainly superoxide, hydroxyl radical, and hydrogen peroxide, have been implicated in carcinogenesis, particularly in the tumor initiation and promotion phases (3). The oxidative damage may occur if the disequilibrium between the production of oxygen free radicals and antioxidative capacity exists and this disequilibrium favors the prooxidant factors. These oxygen radical can be eliminated by superoxide dismutase(SOD) and form part of the hydrogen peroxide in cells. The catalase and glutathione peroxidase(GPX) activities are proven to be inhibited by the superoxide radicals, thus SOD, by which the radicals are scavenged, exerts protective effect on the catalase and GPX. However, the dismutation reaction catalyzed by SOD is under the control of its end product, hydrogen peroxide, which is removed by catalase and GPX, as well. Moreover, it has been noted that tumor cells generally have low levels of MnSOD, CuZnSOD, and catalase, and variable levels of glutathione peroxidase (GPX) when compared with their normal cell counterparts (4). Unlike other tumors, human glioma cells have relatively high levels of MnSOD activity and various levels of CuZnSOD, CAT and GPX (5). These antioxidant enzymes are also known to be a factor in radioresistance and chemoresistance. For example, SOD might play a central role in protecting cells against reactive oxygen species injury during ionizing radiation exposure (7). But, there is few reports dealing with the comparative study of the four main cellular antioxidant enzymes, SOD, CAT, GPX and GPR, in protecting radioresistant variant from radiation injury, especially the parallel comparation in its parent cell line.

It is well known that position in the cell cycle affects radiosensitivity. Generally, cells in S phase tend to be more sensitive than cells in G1, and cells in G2-M are the most sensitive of all. When in exponential growth in the absence of radiation, both cell lines have very similar cell cycle distribution as known in the comparison of growth curve.

The activation of the MAPK pathway by the stress stimulus of ionizing radiation could be due to the fact that double strand DNA breaks lead to different cell cycle checkpoint control. It is reported that the MAPK kinase pathway is required for G2/M progression in a number of systems and ionizing radiation leads both to activation of the MAPK pathway and to G2 arrest. But there is little report that ionizing radiation could activate the MAPK pathway and G2 arrest in the radioresistant system.

In this study, we isolated radioresistant clone(RRC) from U251 human glioblastoma cell by repeated fractions of 3 Gy γ-irradiation , characterized this clone and examined its antioxidant enzymes activities, cell cycle and MAPK compared with U251 parent cell line.

2. Materials and Methods

Cell culture and isolation of radioresistant variant: U251 glioblastoma cell line was obtained from the American Type Culture Collection. Cells were grown in DMEM

(GIBCO/BRL, Grand Island, NY) supplemented with 10% fetal bovine serum. Cell cultures were maintained at at 37℃ in a humidified atmosphere of 5% CO_2. When subconfluent, cultures were exposed to fractionated γ-irradiation (3 Gy per fraction) at room temperature in a ^{137}Cs γ-ray source at a dose rate of approximately 3 Gy/min. A time interval of 3 days was allowed between each fraction to enable the surviving cells to regenerate and this fractionated γ-irradiation was repeated up to 6 months. Seven clones, denoted RRC, were isolated and these clones were radioresistant. RRC7 was the most radioresistant and this clone was used for all experiments.

Clonogenic and apoptotic cell death assay: Clonogenic assay was performed as follows; U251 and RRC in log-phase growth were harvested, resuspended in fresh growth medium, and plated in triplicate in 60-mm dishes at cell concentrations estimated to yield to 20-100 colonies/dish after treatment. Twelve hours after plating, cells were irradiated and cultured for 2 weeks prior to fixation and staining with Coomassie Blue. Colonies with > 50 cells were scored. Apoptotic cell death was examined by DAPI nuclear staining at 24 hrs after 10 Gy radiation.

Western blot and antioxidant enzyme analysis: Western blot analysis was performed after radiation using anti-phosphoMAPK, anti-MAPK and anti-phosphoJNK(New England Biolabs). Total SOD activity was assayed with the method described by Spitz and Oberley (8). One unit of SOD activity is defined as amount of protein required to give half-maximal inhibition of NBT reduction. Catalase activity was measured as described by Claiborne (9). Cell extracts (200-400μg) were added to 3ml of 10 mM H_2O_2 in 50 mM potassium phosphate buffer (pH 7.8) and disappearance of H_2O_2 was immediately measured at 240 nm for 60 sec at 30 sec intervals. Catalase activity was expressed in units per grams of proteins. GPX activity assay was determined as described by Lawrence and Burks (10). Cell extracts (200-400μg/100ul) were incubated in 0.7 ml of a mixture containing 50 mM potassium phosphate buffer (pH 7.8), 1 mM EDTA, 1 mM NaN_3, 1 mM glutathione, 1 U glutathione reductase for 10 min. After addition of 100 μl of 1.5 mM NADPH, NADPH oxidation was measured at 340 nm for 3 min, which is independent of GPX. NADPH oxidation was measured again after addition of 100 μl of 1.5 mM H_2O_2 at 340 nm for 3 min at 20 sec intervals. One unit of GPX activity is defined as the amount of protein required to oxidize 1 μmol NADPH per min. GR was measured according to the method of Paglia and Valentine (11). Enzyme activity was calculated as the number of micromoles of NADPH oxidized per minute per milligram protein using the molar extinction coefficient for NADPH at 340 nm of 6.22 X 106 M^{-1} cm^{-1}. Protein amount was estimated by BCA method.

3. Results and Discussion

One of the major problems in the treatment of gliomas is intrinsic or acquired resistance to radiation. To investigate the radioresistant mechanism, we isolated the radioresistant clone(RRC) by exposing the parent U251 human glioblastoma cell line to repeated fractions of 3 Gy for 6 months, with 3 days recovery allowed between each fractions. In this method, we isolated the radioresistant clone from the parent cells. After 20 passages, there is little different phenotype between RRC and parent cells. Furthermore, as shown in Figure 1A, RRC grows similar to the parent cells. However, when exposed to 5 Gy, RRC had a significant increase in radioresistancy, as measured by clonogenic assay (Figure 1B). To

examine apoptosis directly, both cells were irradiated with 10 Gy, fixed 24 hrs after radiation and stained with DAPI. Figure 1C showed that the apoptosis was much reduced in the RRC compared to the parent cells. A dose of 5 Gy would reduce the clonogenic fraction of the parent cell to approximately 0.05 and the RRC to 0.5 (Figure 1 C). Hence, the difference in the apoptotic fraction was not due to an excess of surviving cells in the RRC. RRC seemed to revert to the wild-type in the absense of radiation over 20 passages, as shown in Figure 1A. However, when exposed to radiation, we observed the conserved radioresistancy in the RRC by clonogenic survival assay in contrast to other report (13). Furthermore, RRC underwent G2/M arrest compared to the parent cells when exposed to radiation, even though the cells showed similar phenotype after long-term culture in the absence of radiation. Therefore, one explanation for this increased radioresistance might be that RRC acquire an adaptive response to radiation. Among the adaptive response to radiation, radical scavenging plays an important role in radioresistancy. Also, cellular superoxide anion production can be increased by radiation and these anions are the likely mediators of tissue injury from oxidative stresses such as ionizing radiation, anticancer drugs and inflammation (14). Therefore, we examined the comparative study of antioxidant enzymes between RRC and its parent lines. The antioxidant enzyme activity in RRC and U251 were investigated in cell lysates after 5 Gy γ-irradiation. As shown in Figure 2A, the basal SOD activity was 1.5 fold higher in RRC. After radiation, the activity increased up to two-folds at 1 hr and remained for 24 hrs. In contrast, no change of SOD activity was observed in U251 parent cell line. Basal catalase activity in RRC was similar to U251 cells, but after radiation, its activity increased up to 1.7 fold in RRC at 10 hrs (Figure 2 A). In parent cell, the activity slightly decreased at 1 hr after radiation and conserved for 24 hrs. Increased activity of SOD and catalase in RRC correlates the radioresistancy after radiation. However, SOD and catalase activation after radiation were different in time course. SOD activation was earlier than catalase. As oxygen radical is converted to hydrogen peroxide by SOD, it is important to remove radiation-induced radical rapidly. Then, catalase consumes hydrogen peroxide catalyzed by SOD. Therefore, earlier activation of SOD may play a role in protecting RRC from radical injury. GPX activity, which can remove hydrogen peroxide using glutathione, increased approximately 3.7 folds in basal level and further activated up to 5 folds higher than U251 after radiation (Figure 2 A). Furthermore, GPX was activated about 1 hr after radiation. However, in parent cell line, GPX level was relatively low and activated up to 1.6 fold at 10 hrs after radiation. This suggests that hydrogen peroxide produced by SOD might be mainly utilized by GPX rather than catalase. As shown in figure 2A, following radiation, GR activity in RRC increased as the same in the case of SOD. In contrast, in parent cells, its activity was not changed after radiation. GR regenerates the reduced glutathione using NADPH and cellular glutathione redox level depends on the cellular redox state. It is proposed that GR activity might be correlated with SOD in radioprotection. Very recent studies showed that cytosolic GPX overexpression was insufficient to confer radioresistance in CHO and Sup-T1 cell lines (15). However, it is important that a single antioxidant enzyme alone can not be a determinant of protecting radiation-induced injury.

To examine the difference of MAPK activation, we performed Western blot analysis in both cells after 5 Gy radiation. As shown in Figure 2B, RRC has a higher activation of Erk 1/2 than that of U251. In case of JNK, there is little difference in both cells. However, Erk1/2 activation in RRC was not conserved compared to U251. Since it is reported that MAPK

pathway is necessary for progression through G2/M progression, our results suggests that higher activation is required for radioresistancy and prolonged activation is prerequisite for the G2/M progression respectively.

In conclusion, we isolated the radioresistant clone from U251 human glioblastoma cell by exposing repeated 3 Gy fraction. RRC had a higher radioresistancy compared to the parent cell line and a delayed G2 arrest after radiation. RRC showed higher activation of Erk1/2 not JNK. Therfore, this radioresistancy was involved in the delayed cell cycle, higher level of activated Erk1/2 and a coordinated high level of antioxidant enzyme activities, that is, early activation of SOD, GR and GPX, and later in catalase.

4. Acknowledgement

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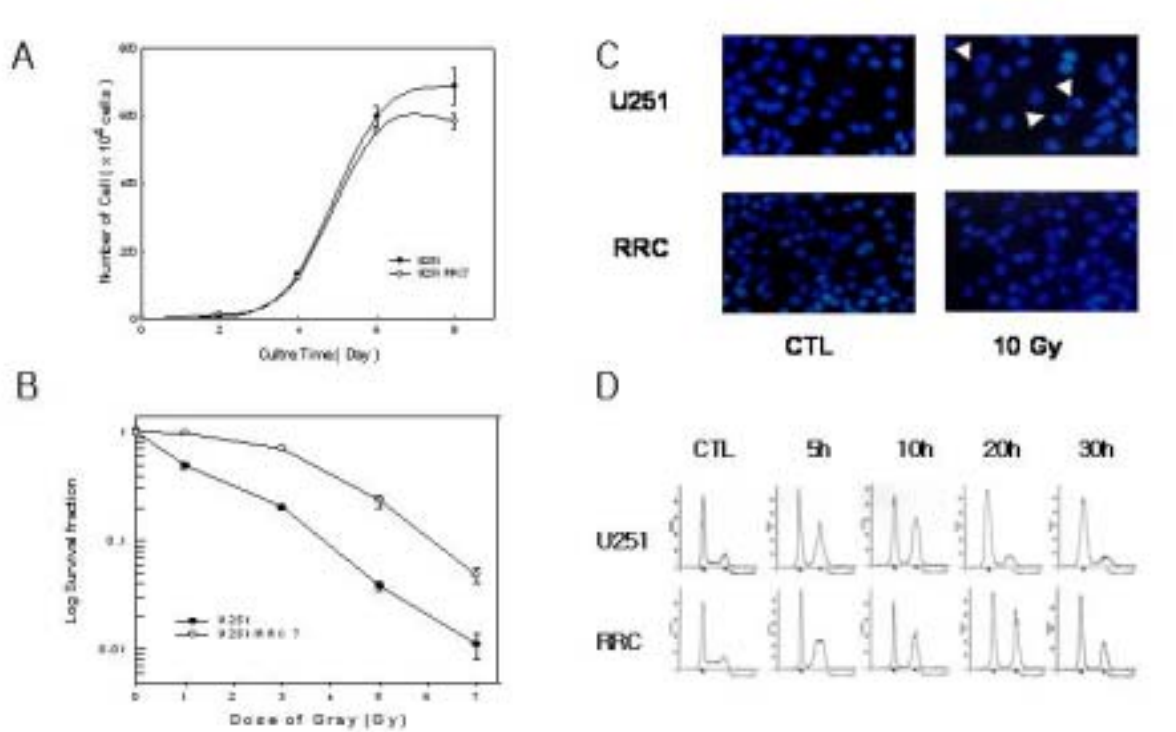

Figure 1. Growth curve (A), clonogenic survival assay (B), DAPI staining (C) and FACS

analysis(D) of U251 human glioblastoma cell line and RRC. For clonogenic assay, twelve hours after plating, cells were radiated and cultured for 2 weeks prior to fixation and staining with Coomassie Blue. DAPI staining was performed after 24 hrs of 10 Gy radiation. Arrow heads(C) indicate the apoptotic cells. FACS analysis was performed after 3 Gy radiation at the indicated time.

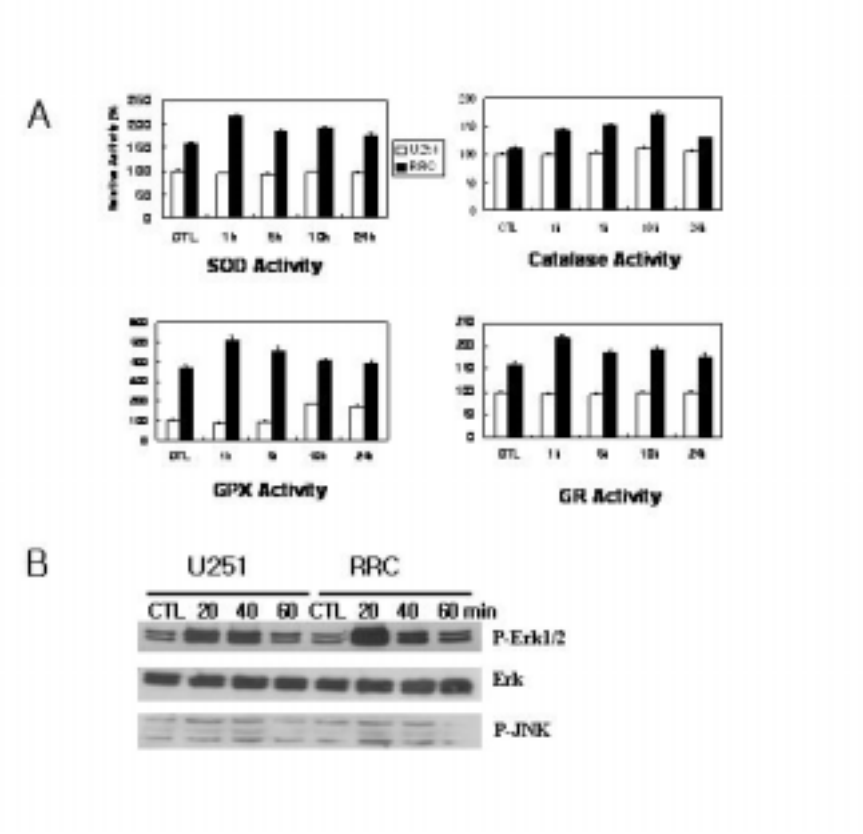

Figure 2. Comparison of antioxidant enzymes between U251 and RRC. After 5 Gy radiation, cells were harvested at the indicated time and antioxidant enzymes assays were performed for SOD and catalase (A), GPX and GR (B). Western blot analysis was performed at the indcated time after 5 Gy radiation.

5. References

1 Davis, L.W., Malignant glioma--a nemesis which requires clinical and basic investigation in radiation oncology, Int. J. Radiat. Oncol. Biol. Phys., 16 (1989) 1355-1365.

2 Yount, G.L., Haas-Kogan, D.A., Levine, K.S., Aldape, K.D. and Israel, M.A., Ionizing radiation inhibits chemotherapy-induced apoptosis in cultured glioma cells: implications for combined modality therapy, Cancer Res., 58 (1998) 3819-3825.

3 Cerutti, P.A., Prooxidant states and tumor promotion, Science, 227 (1985) 375-381.
4 Oberley, L.W. and Buettner, G.R., Role of superoxide dismutase in cancer: a review, Cancer Res., 39 (1979) 1141-1149.
5 Zhong, W., Yan, T., Lim, R. and Oberley, L.W., Expression of superoxide dismutases, catalase, and glutathione peroxidase in glioma cells, Free Radic. Biol. Med., 27 (1999) 1334-1345.
7 Sun, J., Chen, Y., Li, M. and Ge, Z., Role of antioxidant enzymes on ionizing radiation resistance, Free Radic. Biol. Med., 24 (1998) 586-593.
8 Spitz, D.R. and Oberley, L.W., An assay for superoxide dismutase activity in mammalian tissue homogenates, Anal. Biochem., 179 (1989) 8-18.
9 Claiborne, A., Catalase activity. In: Greenwald, R. A., edHandbook of methods for oxygen radical research Boca Raton, FL: CRC Press Inc., (1985) 283-284.
10 Lawrence, R.A. and Burk, R.F., Glutathione peroxidase activity in selenium-deficient rat liver, Biochem. Biophys. Res. Commun., 71 (1976) 952-958.
11 Paglia, D.E. and Valentine, W.N., Studies on the quantitative and qualitative characterization of erythrocyte glutathione peroxidase, J. Lab. Clin. Med., 70 (1967) 158-169.
12 Bernhard, E.J., Maity, A., Muschel, R.J. and McKenna, W.G., Effects of ionizing radiation on cell cycle progression. A review, Radiat. Environ. Biophys., 34 (1995) 79-83.
13 Russell, J., Wheldon, T.E. and Stanton, P., A radioresistant variant derived from a human neuroblastoma cell line is less prone to radiation-induced apoptosis, Cancer Res., 55 (1995) 4915-4921.
14 Freeman, B.A. and Crapo, J.D., Biology of diseases. Free radicals and tissue injury, Lab. Invest., 47 (1982) 412-426.
15 Mansur, D.B., Kataoka, Y., Grdina, D.J. and Diamond, A.M., Radiosensitivity of mammalian cell lines engineered to overexpress cytosolic glutathione peroxidase, Radiat. Res., 155 (2001) 536-542.