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Cell Cycle Checkpoint and Apoptosis Induction in Glioblastoma Cells and Fibroblasts Irradiated with Carbon Beam

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Glioblastoma/High LET Charged Particle/*p53*/G2 block/Apoptosis.

This study was conducted in order to evaluate the cytotoxicity of high linear-energy-transfer (LET) ionizing radiation (IR) on glioblastoma cells and fibroblasts using different modes of cell inactivation assays. Two human glioblastoma cell lines with or without *p53*-mutation, and fibroblasts were used as materials. Gamma rays and 290 MeV/u carbon beams with LET values of 20, 40, 80 keV/μm were used. To evaluate cell inactivation, we used colony formation assay, morphological detection of apoptosis, and flow-cytometry. Serial expressions of p53 and p21 were analyzed by immunoblotting. High-LET IR reduced the reproductive potency of these cells to identical levels in spite of differences in gamma-sensitivity, and yield of cell death correlated to LET values. A *p53*-wild- type glioblastoma cell line demonstrated a higher yield of apoptosis than other cell lines, whereas fibroblasts hardly displayed any cell death indicating senescence-like growth arrest even after high LET IR. A *p53*-mutant tumor cell line demonstrated very low yield of cell death with prominent G2/M arrest. Results of radiosensitivity differ according to what mode of cell inactivation is selected. While fibroblasts depend on G1 block after IR, G2/M blocks may play crucial roles in the radioresistance of *p53*-mutant glioblastoma cells.

INTRODUCTION

Accelerated heavy ion beams have been tested for their effectiveness on the basis that they can provide a greater localization of energy to the targeted volume as well as greater cell-killing potency.[1–3] Based on this promising advantage, it has been a great concern that high linear-energy-transfer (LET) particle beams can overcome radioresistance of tumors such as glioblastoma which has been known as one of the most radio-resistant tumors.

Radiation induced cell inactivation encompasses very wide-ranging issues. Loss of reproductive activity has been used widely to evaluate radiosensitivity of mammalian cells as clonogenic survival assay, and in the same line, a concept of senescence-like growth arrest has been advocated recently.[4,5] This phenomenon is defined as irreversible growth arrest of cells after ionizing radiation (IR) with morphological changes consisting of flattering and increased granularity, and its molecular mechanism has been partly elucidated.[6] In addition, radiation induced cell death, in terms of cell destruction, is now mainly classified into three modes. Apoptosis, type-I cell death, is a caspase dependent programmed cell death which is morphologically characterized by chromatin condensation and nuclear fragmentation. Autophagic cell death, type-II cell death, is considered to be another mode of programmed cell death, which is characterized by increased number of autophagosomes in the cytoplasm.[7–9] In contrast to these programmed cell deaths, necrosis, type III cell death, is regarded as passive form of unregulated process of cell destruction presenting marked cell swelling followed by release of intracellular components.

On the other hand, cell inactivation induced by IR with different LET's has been analyzed so far, and many studies have shown that high LET IR of up to approximately 100 keV/ μm is more effective in cell killing and mutation induction than low LET IR.[1–3,10,11] More recently, a similar experimental result was reported in V79 cells both in yield of

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apoptosis and reproductive death,[12] and also, high LET IR is reported to enhance apoptosis in lung cancer cells regardless of *p53* status.[13] As compared to low LET IR, high LET irradiation induce more complex clustered damages to DNA with reduced repairability, which is considered to be the unique mechanism as a determinant of RBE of high LET IR.[14]

As for glioblastoma cells, reports on the issue of cell death induced by low LET[5,15,16] or high LET IR[17,18] are still limited to our knowledge. Under these circumstances, here we report the results of *in vitro* analysis on not only the reproductive activity but also the incidence of cell death in correlation with cell cycle changes after exposure to IR with various LET in both tumor and normal cells. By elucidating the radiosensitivity of glioblastoma cells against high LET IR, we will be able to find clues to help us to achieve higher therapeutic gain in glioblastoma patients.

METHODS AND MATERIALS

Cell lines and cell culture condition

Glioblastoma cell lines: TK1,[19] U87MG[20] and normal human fibroblast: NB1RGB[21] were used as materials. It has been confirmed that TK1 has a transition in codon 273 in exon 8 of *p53* gene, while NB1RGB, which was purchased from RIKEN Cell Bank, has been widely used as normal control cells.[22,23] Also, U87MG has wild-type *p53* as mentioned in various literatures.[24,25] The cell culture conditions were as mentioned previously.[10,17] Briefly, cells were cultured in minimum essential medium (MEM) supplemented with 100 μg/ml streptomycin, 100 U/ml penicillin and 10% fetal calf serum (FCS). The cultures were maintained at 37°C in a humidified incubator with 5% CO_2 tension. During subculturing, the cells were rinsed once with calcium- and magnesium-free phosphate-buffered saline (PBS) and exposed to a 0.25% trypsin solution containing 0.5 mM EDTA. Cells in flasks before reaching confluence were used in these experiments.

Irradiation

Accelerated mono-energetic carbon ions of 290 MeV/u were generated by heavy ion medical accelerator (HIMAC) at National Institute of Radiological Sciences (NIRS) in Chiba, Japan as mentioned before.[26] Approximately 20, 40, 80 keV/μm of LET's were selected in this study. Comparative experiments were performed using ^{137}Cs gamma rays (1 keV/μm) at a dose rate of approximately 1.2 Gy/min.[27] In all radiation qualities, 5 dose points were selected as indicated for clonogenic survival analysis. Tumor cells in the flask were observed by eyes and irradiated before they reached a confluent state. As for apoptosis detection, our previous experiments indicated that fibroblasts or glioblastoma cells do not undergo apoptosis at doses from 1 to 8 Gy, which are usually used in clonogenic survival assay. Therefore, a single physical dose of 10 Gy was given to cells for cell death assays except for clonogenic survival assay in this study. Further details of the irradiation conditions, dosimetry and LET measurement procedures are mentioned elsewhere.[26]

Clonogenic survival assay

Standard colony formation assay was performed immediately after exposure to carbon beams or gamma rays. Following irradiation, cells in the monolayer culture were trypsinized and suspended in PBS on ice until plating into 60 mm dishes for colony formation. The colonies were fixed and stained after approximately 20 days. Five replicate dishes were seeded for each dose point, and colonies containing more than 50 cells were scored as survivors. Survival curves were fitted by the linear-quadratic (LQ) model using a software DeltaGraph v.5.4 (Deltapoint, Polaroid Japan, Tokyo) and the dose corresponding to 10% survival of each cell was used to calculate relative biological effectiveness (RBE). Three independent experiments were performed on each LET value including gamma rays. Average plating efficiencies of these three cell lines were as follows; 61% in TK1, 35% in U87 MG and 18% in NB1RGB.

Morphological detection of apoptotic cells

On days 1, 4, 7 and 10 following irradiation, cells in the media as well as cells adhering to the bottom of flasks were collected by trypsinization and centrifuged at an average of 327 × g for 5 minutes. In order to salvage as many degraded fragments as possible, media was not changed during post-irradiation incubation period. Then, the cells were washed by PBS and fixed by 2% glutaraldehyde in PBS at 4°C overnight. The fixed cells were stained by Hoechst 33342 at a final concentration of 1 mM. After incubation for five minutes, the morphological change of the nucleus was observed, and the number of apoptotic cells and total number of cells were counted with a fluorescent microscope. The apoptotic index (AI) was calculated as percent value of apoptotic cells against total number of cells. Three independent experiments were performed and the average values were plotted against time.

Flow-cytometry analysis

On days 1, 4, 7 and 10 following irradiation, fragments in the media and the trypsinized cells were collected by centrifuging at an average of 327 × g for 15 minutes and washed once by PBS. The cell pellets were gently resuspended in 100 μl of 0.9% NaCl, which was slowly dripped into 2 ml of ice-cold 70% ethanol with vigorous agitation. These cells in 70% ethanol were stored at 4°C over-night for fixation. Just before flow-cytometric analysis, fixed cells were centrifuged and rinsed by PBS with 0.5% Tween-20. Then final cell pellets were resuspended in 1 ml of PBS containing 5 μg/ml of propedium iodide (PI), and incubated on ice for

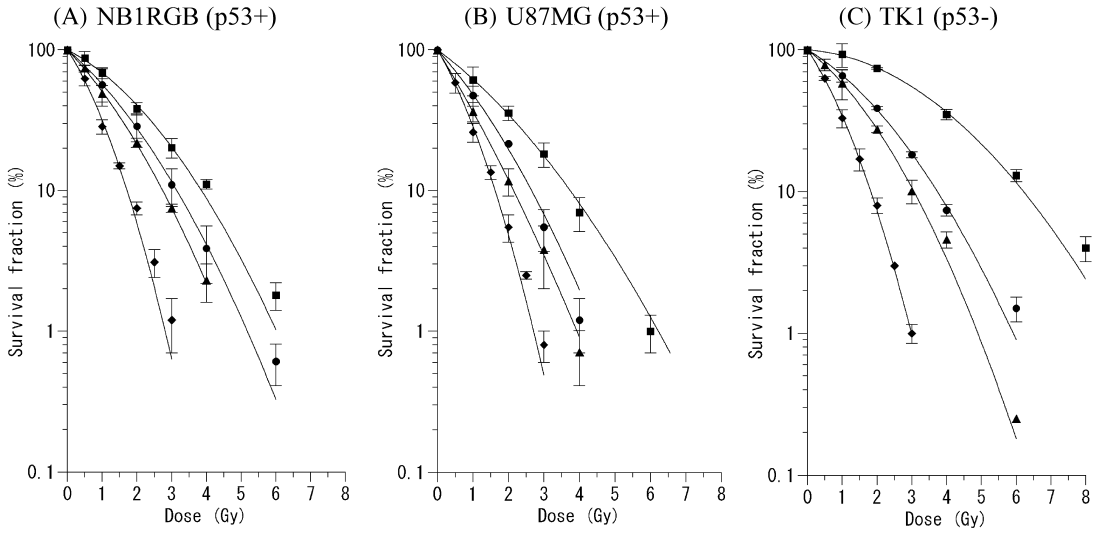

Fig. 1. Clonogenic survival curves of (A) NB1RGB, (B) U87MG and (C) TK-1. Symbols of ■, ●, ▲ and ◆ represent gamma rays, 20, 40 and 80 keV/μm carbon beams, respectively. Data points are fitted by the linear-quadratic model, and error bars indicate standard deviations.

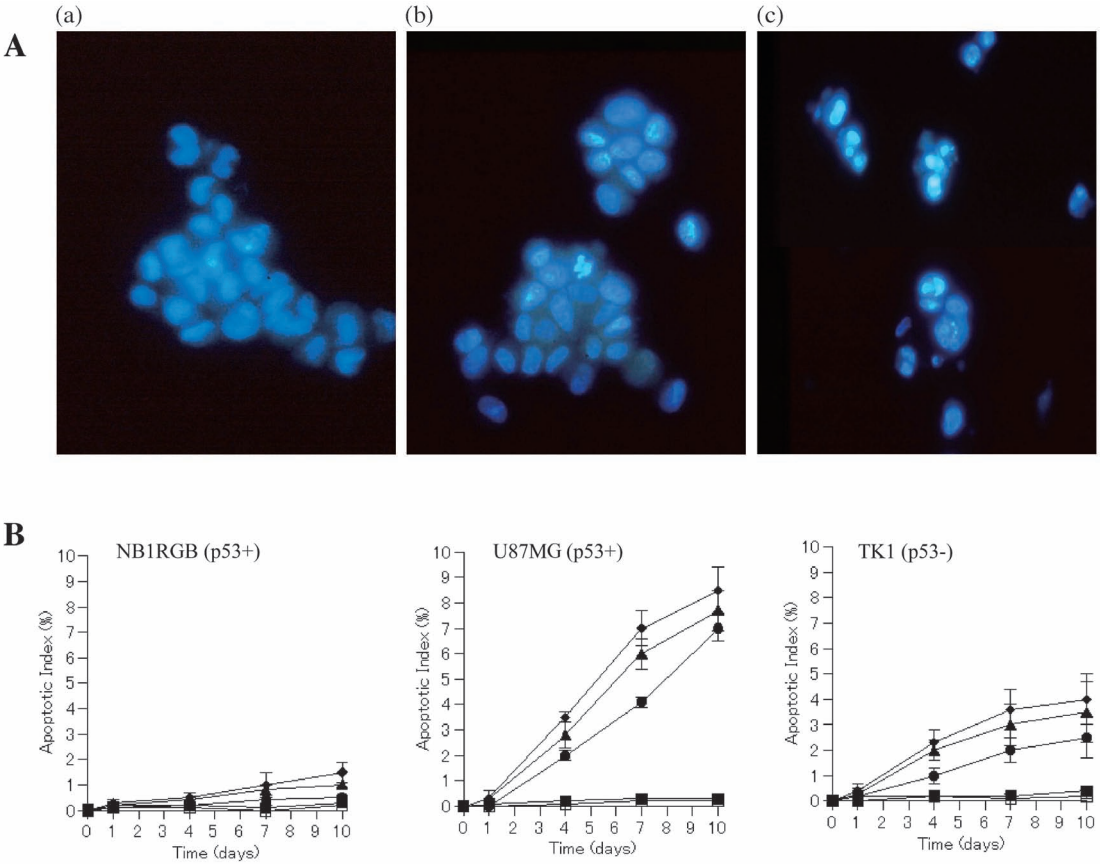

Fig. 2-A: Fluorescent microscopic observations of U87MG cells stained with Hoechst 33342 before irradiation (a), 1 day (b) and 7 days (c) after irradiation with 10 Gy of 80 keV/μm carbon beam. Chromatin condensation and fragmentation were observed in (c).
2-B: Apoptotic index (AI) calculated as percent number of morphologically detected apoptotic cells as shown in Fig. 2-A. Cells were irradiated with a dose of 10 Gy in every quality of radiation. Symbols of ■, ●, ▲, ◆ and □ represent gamma rays, 20, 40, 80 keV/μm carbon beams and control (no irradiation), respectively. Error bars indicate standard deviations.

approximately 30 minutes. The sub-G0/G1, S and G2/M phase fractions of each cell line were obtained from DNA histograms by FACScaliber (Beckton Dickinson Japan, Tokyo) using a cell cycle analysis software Modofit (Beckton Dickinson Japan, Tokyo) following the manufacturer's instructions. Two independent experiments were performed.

Immunoblotting

Total protein was extracted from cells by lysis buffer consisting of 50 mM Tris-HCl pH 8.0, 150 mM NaCl, 1% NP-40, 5 mM EDTA, 1 mM PMSF, 1 µg/ml Pepstatin, 2 µg/ml Aprotinin, 0.5 µg/ml leupeptin, 1mM Dithyothreitol (DTT) before and on day 1, 4, 7 and 10 after irradiation. After protein concentration of the material was determined using Protein Assay Kit (BioRad Japan, Tsukuba) according to the manufacturer's protocol, approximately 30 µg of each protein was fractionated through 12% polyacrylamide gel electrophoresis. Fractionated protein in the gel was transferred to nitrocellulose membrane (BioRad Japan, Tsukuba) by semi-dry western blotting technique using Transblot (BioRad Japan, Tsukuba). Membranes were first probed by anti-p53 monoclonal antibody (DAKO, Tokyo), and anti-p21 monoclonal antibody (Santa Cruz Biotech, California) followed by secondary anti-mouse IgG antibody HRP conjugated (BioRad Japan, Tsukuba). Bands were visualized by ECL system (Amerciam-Pharmacia Biotech Japan, Tokyo).

RESULTS

Colony formation assay

The results of colony formation assay are shown in Fig. 1. The error bars on the survival curves indicate standard deviation calculated from 3 independent experiments. While typical shoulders were noticed on gamma survival curves, carbon beam-induced survival curves displayed nearly exponential dose responses. A glioblastoma cell line with *p53*

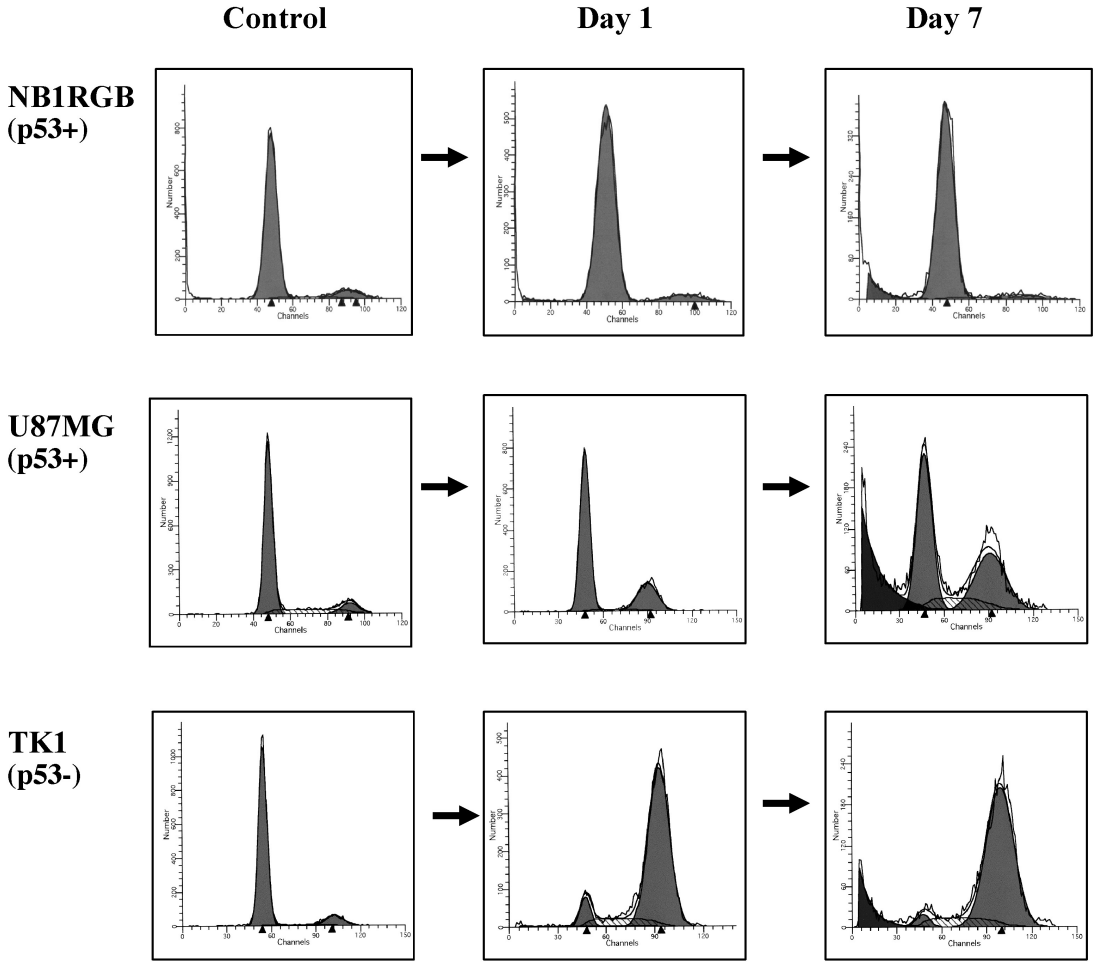

Fig. 3. Representative results of serial DNA histograms of three cell lines obtained after 10 Gy of 80 keV/µm carbon-beam irradiation are demonstrated. In fibroblasts (NB1RGB), G2/M accumulation is hardly seen and yield of apoptosis is very small. In contrast, *p53* mutant tumor cells (TK1) demonstrated a prominent G2/M accumulation. The degree of G2/M accumulation in *p53* wild-type tumor cells (U87MG) lies between the other two and the yield of apoptosis is the highest among these.

mutation, TK1, is most resistant to IR of every quality while fibroblast cells NB1RGB and *p53* wild cell line U87MG show almost equal sensitivities. The slopes of these cell lines became closer to each other as LET value increases; and at 80 keV/μm, survival curves of three cell lines are almost identical. RBE of carbon beams of 20, 40 and 80 keV/μm calculated from these survival curves were 1.2, 1.5, 2.4 in NB1RGB, 1.4, 1.8, 2.5 in U87MG, and 1.7, 2.1, 3.6 in TK1, respectively. These results may indicate that RBE of high LET IR measured by this assay becomes greater in gamma resistant tumor cell lines.

Detection of apoptotic cells

Apoptotic cells were detected under fluorescent microscope as shown in Fig. 2-A. The calculated serial values of apoptotic indexes (AI) are shown in Fig. 2-B. Apoptotic cells were not clearly observed on day 1 in any cell line, and were hardly observed after gamma irradiation in any cell line even

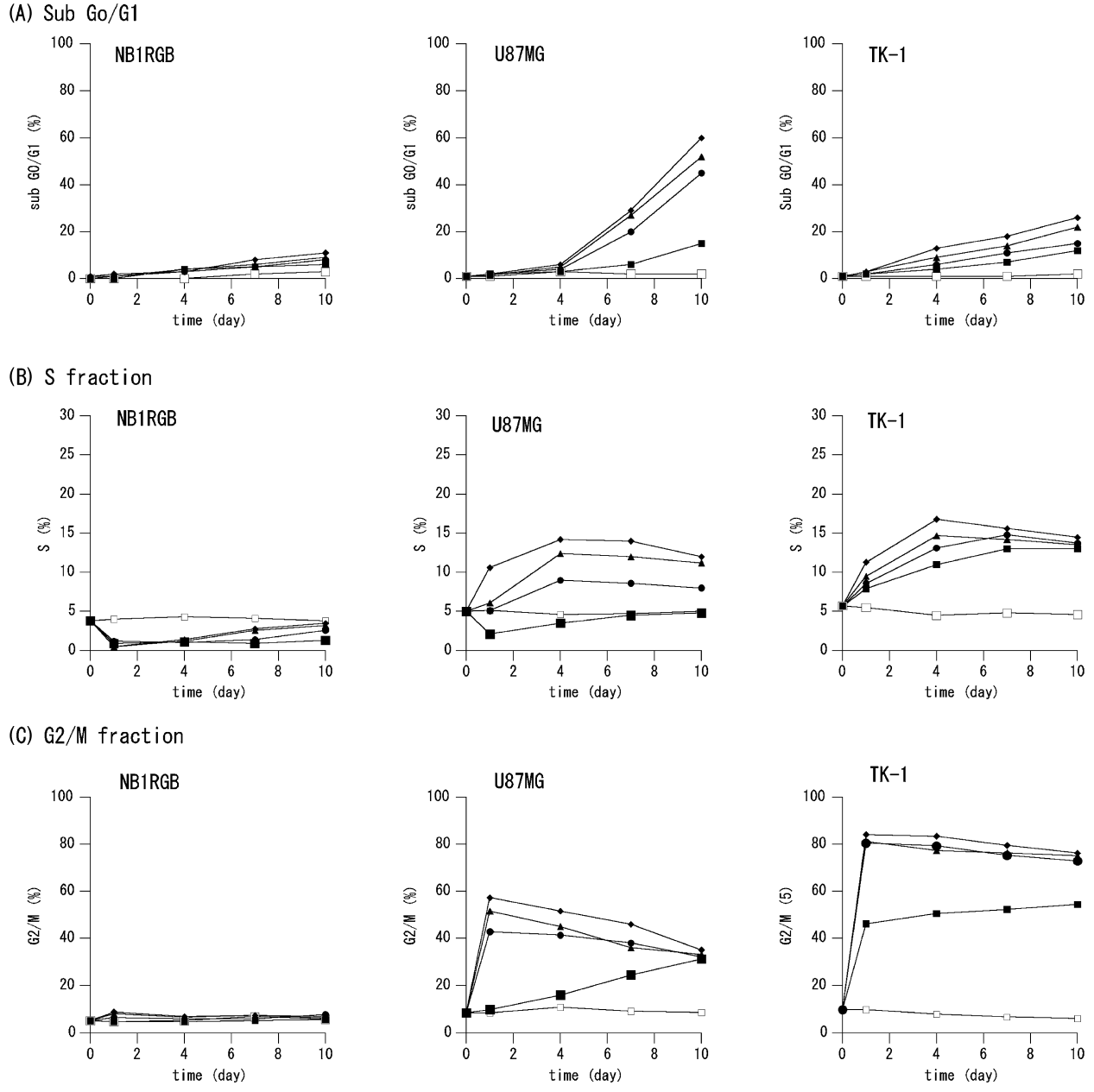

Fig. 4. Serial change of (A) sub G0/G1, (B) S and (C) G2/M fractions after irradiation in NB1RGB, U87MG and TK-1. Symbols of ■, ●, ▲, ◆ and □ represent gamma rays, 20, 40, 80 keV/μm carbon beams and control (no irradiation) respectively. G1 fractions are not contained in this Fig.

after 10 days of incubation. In high LET IR, the yield of apoptosis correlated with LET values and incubation period in all cell lines. The maximum AI was obtained on day 10 after 80 keV/μm carbon beam irradiation in every cell line. U87MG demonstrated the highest and NB1RGB demonstrated the lowest incidence of apoptosis. Especially, NB1RGB showed a very low level of AI even after 80 keV/μm carbon beam irradiation. The maximum AI in NB1RGB, U87MG and TK1 were 1.5%, 4.0% and 8.5%, respectively.

Flow-cytometry (FCM)

Representative results of serial DNA histograms of three cell lines obtained after 80 keV/μm carbon beams are shown in Fig. 3. In addition, the average values of sub G0/G1, S and G2/M fractions were calculated from two independent experiments and plotted against time in Fig. 4.

Sub G0/G1 fraction did not appear on day1 after any irradiation, but increased in correlation with time and LET value in every cell line. It was indicated that NB1RGB showed significantly smaller sub G0/G1 fractions than two tumor cell lines, and U87MG demonstrated a higher yield of sub G0/G1 fractions than TK1 (Fig. 3, Fig. 4-A). These are compatible with the results of AI demonstrated above (Fig. 2-B).

The S-phase fraction decreased concomitant with increased accumulation in the G1-phase in NB1RGB after every irradiation, while it increased in a LET dependent manner in both TK1 and U87MG (Fig. 3, 4-B). In contrast, NB1RGB demonstrated a very small G2/M fraction increase even after high LET IR, while G2/M accumulation was very prominent in TK1 after gamma ray or carbon beam irradiation. U87MG also demonstrated remarkable G2/M accumulation after high LET IR, but its degree was less than TK1 (Fig. 3, Fig. 4-C).

Immunoblotting

Serial expression of p53 and p21 after irradiation in three cell lines were analyzed by methods mentioned above. Normal human skin fibroblast cells NB1RGB and *p53* wild-type glioblastoma cell line U87MG showed increases of p53 expression after irradiation associated with simultaneous up-regulation of p21 (Fig. 5). In NB1RGB, high levels of expression were maintained for a longer period of time as LET increased from gamma to 80 keV/μm carbon beam (Fig. 5). In contrast, U87MG exhibited a reverse tendency in which the expression of both p53 and p21 gradually decreased as LET increased (Fig. 5). In a *p53* null glioblastoma cell line TK1, expression of p53 constantly maintained a very high level before and after every irradiation, and there was no expression of p21 (Fig. 5).

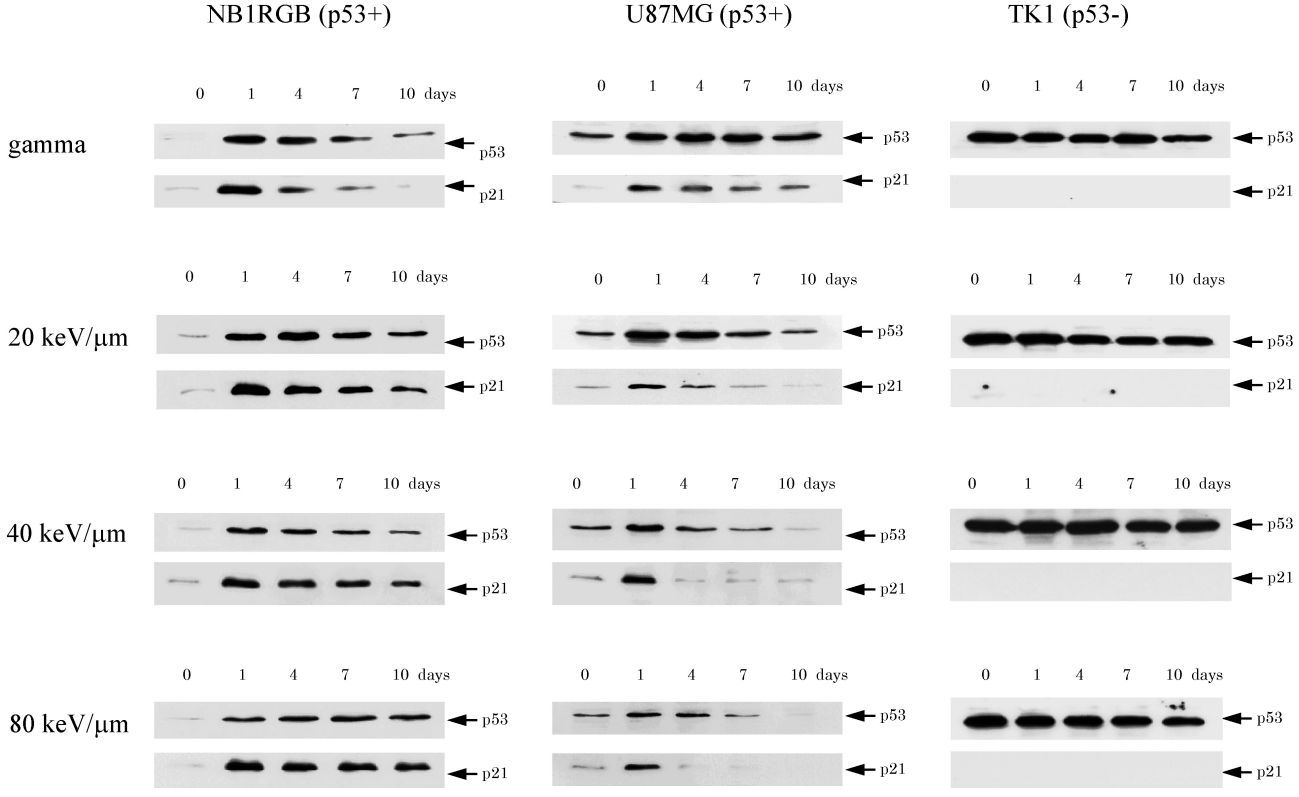

Fig. 5. Immunoblotting demonstrating chnological changes of p53 and p21 expressions after irradiation with 10 Gy of gamma rays, 20, 40 and 80 keV/μm carbon beams in NB1RGB, U87MB and TK1.

DISCUSSION

It has been reported that rapid apoptosis is unlikely to occur in glioma cell lines after low-LET radiation.[16] Later, Yao *et al*. reported that apoptosis did not occur in 6 glioblastoma cell lines following gamma irradiation. Instead, they observed autophagic cell change.[15] Based on these, it is becoming clear that apoptosis does not play a significant role in the killing of glioblastoma cells after IR at least with low-LET. As for high-LET IR, although *in vitro* cytotoxicity of carbon beams against glioblastoma cells has been analyzed by standard clonogenic survival assay[17,18] or detection of apoptotic cells,[15,18] the whole scheme of glioblastoma cell killing after high-LET IR remains to be cleared. Thus further detailed analyses have been expected to search for clues to increase radiosensitivity of glioblastoma cells.

There are several known concepts on radiation induced cell death in relation to cell cycle progression. Interphase death is used when cell death occurs before they reach the first mitosis; in contrast, mitotic death indicates cell death after one or two divisions.[28,29] Mitotic catastrophe is a rather new concept of cell death in which cells are destroyed at mitosis to prevent cells from aneuploidization.[30] On the other hand, IR induced cell death, in terms of cell destruction, has been analyzed by mainly morphological studies, and now it encompasses three modes of cell death as mentioned in the introduction part.

Colony formation assay measures the number of cells maintaining reproductive or clonogenic potency after IR, and it may reportedly demonstrate a combination of interphase and reproductive death.[29] However, our preliminary experiments indicated that fibroblasts or glioblastoma cells do not undergo apoptosis at doses from 1 to 8 Gy. This indicates that colony formation assays demonstrate only the reproductive potency after IR of this dose rang in our cell lines. TK1 demonstrated the highest resistance to gamma rays, and NB1RGB and U87MG showed almost equal sensitivities, survival curves of these cell lines became closer and straight as LET increased. This was compatible with our previous study using different glioblastoma cell lines,[17] indicating that high LET IR may reduce the reproductive potency to the almost identical level in spite of the difference in gamma sensitivity.

In addition to clonogenic survival assays, we applied two methods to evaluate cell death including morphological detection of apoptosis and flow-cytometry using a single dose of 10 Gy at which reproductive proficiency is hardly expected in these cell lines. Morphological observation of the nucleus stained by Hoechst 33342 is an accepted method to detect apoptosis, and this method may not detect small DNA fragments at later phases of the apoptotic process[31] with the centrifuge condition of 327 × g for 5 minutes. In contrast, flow-cytometry can quantitatively detect various sizes of degraded DNA fragments resulting from all modes of cell death[32] after centrifuge for 15 minutes.

Gamma rays hardly induced apoptosis in these cells. In contrast, after high LET irradiation, the incidence of apoptosis increased as LET increased in every cell line, although the maximum AI values were smaller than we initially expected. Similar LET dependency is observed in the flow-cytometry analysis with a propensity that a *p53* positive tumor cell line (U87MG) showed a higher yield of sub G0/G1 fraction than a *p53* negative tumor cell line (TK1) or fibroblasts (NB1RGB). The yield of cell death demonstrated in the flow-cytometry analysis was approximately five times greater than AI, which might partly result from the difference in centrifuge period indicated above. Further analysis on the morphological changes including autophagy and mitotic catastrophe should be expected.

As shown so far, radiosensitivity measured by these cell death assays may differ from that measured by reproductive inactivity using colony formation assay. For example, at 80 keV/μm, the clonogenic survival curves are almost identical in these cell lines, but the yield of apoptosis or sub G0/G1 fraction differed significantly. Similarly, all cell lines scarcely became apoptotic after gamma ray irradiation in spite of their remarkable difference in gamma survival in clonogenic assay. Although the loss of reproductive potency and cell death may be on the same line, it is conceivable that less genetic injury is required for loss of reproducibility in cells than for other modes of cell death after IR, and other mechanisms in repair potency as well as the status of *p53* may explain these differences in sensitivity. As for further analysis, it is recommended to evaluate yield of different modes of cell death after various irradiation at doses that induce the same clonogenic survival fraction. Therefore, not only clonogenicity but also other modes of cell death should be measured in order to evaluate radiosensitivity of cells more precisely.

After exposure to IR, mammalian cycling cells are arrested at G1 check-point *p53* associated with up-regulation of p21 which inhibits cell cycle promoter Cdk2/Cyclin-E,[33] and they are also arrested at G2/M check-point by activation of Chk1 and Chk2 which may phosphorylate Cdc25C to inhibit Cdc2/Cyclin-B and prevent entry into mitosis.[34] Thus, U87MG and NB1RGB demonstrated G1 arrest associated with increased levels of p53 and p21 expression after gamma irradiation. However, serial expressions of p53 and p21 after high LET irradiation are remarkably different between U87MG and NB1RGB; their expressions significantly decreased with time and in a LET-dependent manner in U87MG, whereas they were expressed continuously in NB1RGB. These are compatible with our data that G1 arrest was not demonstrated in U87MG after carbon beam irradiation with LET of 40 keV/μm or higher.

As the incidence of apoptosis in U87MG increased in spite of decreased expression of p53 after high LET radia-

tion, *p53* may become less irrelevant to the yield of apoptosis as LET increased in this cell line. These facts may indicate that function of *p53* in NB1RGB may be different from that in U87MG. This phenomenon strongly suggests senescence-like growth arrests in NB1RGB as a *p53*-dependent event following IR[4,6] and/or resulted from synchronization in G1 due to density inhibition. These are also compatible with the results of other studies on human fibroblasts. Hamada *et al*. mention that delayed reproductive death occurred in a dose-dependent manner,[11] and Azzam *et al*. observed lack of reduction in the fraction of cells permanently arrested on G1 after alpha particle irradiation.[35] Further analyses on not only morphological observation but also expression of beta-galactosidase should be planned on NB1RGB to confirm this speculation. In contrast, TK1 demonstrates marked and prolonged G2/M block without p21 expression after high LET irradiation. U87MG also showed G2/M block in a similar pattern by a smaller degree. Thus, we may observe the balance of two major protective cell cycle check point mechanisms, G1 and G2/M blocks. The former may play a major role in NB1RGB while the latter may in TK1 and both are playing to some limited degrees in U87MG. However, as not only *p53* status but also other genetic backgrounds are unlikely to be consistent between the two glioblastoma cell lines, a role of *p53* proposed in this report is not considered to be very conclusive. In addition, IR-induced G1 arrest in U87MG is nevertheless much less pronounced than in fibroblasts, implying that *p53* downstream effectors and/or other checkpoint-related genes may be impaired in U87MG. In the most resistant cell line TK1, sub G0/G1 fractions in the flow-cytometry analysis is very small whatever process of cell death may occur. This is compatible with the observation that there is a correlation between G2/M block and radiosensitivity in some tumor cell lines.[17,36] Therefore, it is likely that modulation focused on the G2/M check points is a good strategy for improving radioresistance of glioblastoma cells after high LET IR.

In conclusion, our data demonstrated that accelerated carbon beams could yield cell death including apoptosis in glioblastoma cell lines in a LET dependent manner, and clonogenic survival was compatible with the previous reports. It was indicated that results of radiosensitivity may significantly differ according to what mode of cell death is selected as the endpoint. Owing to the sustained G1 block associated with p53 and p21 expression, the yield of cell death in fibroblasts was significantly smaller than other tumor cell lines even after exposure to high LET IR. In contrast, glioblastoma cells harboring wild-type *p53* demonstrated both G1 and G2/M blocks to limited degrees yielding a large amount of cell death, while *p53*-null tumor cells demonstrated prominent G2/M block; which may be responsible for the marked radioresistance. These basic findings may indicate that high-LET IR with molecular targeting focused on the modulation of G2/M check point specifically increases radiosensitivity of glioblastoma cells, which can be a good candidate in future therapeutic strategies in patients with glioblastoma.

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