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The Effect of Carbon Ion Beam Irradiation for Hypoxia-Mediated Invasion of Glioblastoma

Kazumasa MINAMI¹, Masahiko KOIZUMI², Yoshinosuke HAMADA¹,³,⁴, Seiji MORI¹, Naomasa KAWAGUCHI¹, Masashi MANABE⁴, Hiromasa IMAIZUMI², Kana NAKATANI², and Nariaki MATSUURA¹

¹Department of Functional Diagnostic Science, ²Department of Medical Physics and Engineering, ³Department of Health Economics and Management, Osaka University Graduate School of Medicine, Osaka, Japan ⁴Department of Pediatric Dentistry, Osaka Dental University, Osaka, Japan

Synopsis

Glioblastoma is one of the most fatal malignant tumors because of its invasiveness and radioresistance. Unfortunately photon irradiation and hypoxic condition are reported to enhance cancer cells’ aggressiveness respectively. The purpose of this study is to clarify the effect of carbon ion beam on invasive potential of glioblastoma cells. Relative Biological Effectiveness of A172 cells measured by the dose for 1% survival fraction was 2.22. X-irradiation and hypoxic condition promoted invasion and migration of A172 and T98G cells respectively, while carbon ion beam suppressed both the two capabilities enhanced by hypoxia. GTP-RhoA (active form) was increased by 0.5 Gy of X-irradiation. On the other hand RhoA was inactivated by 1 and 4 Gy of carbon ion beam. Carbon ion beam irradiation suppressed glioblastoma cells’ invasion and migration via inactivation of RhoA. Furthermore carbon ion beam also inhibited these abilities enhanced by hypoxia.

Key words: Carbon ion beam, invasion, glioblastoma

Introduction

In these decades, while the local control rate of the malignant tumor has improved by the highly accurate radiation therapy, cancer-related mortality has risen every year. Both operation and radiotherapy are the local treatments and not effective against metastasizing cancer. Therefore, metastasis of cancer is considered a crucial issue that should be overcome.

Glioblastoma multiform (GBM) is one of the most fatal malignant tumors. Typically the boundary of its invasion is indistinguishable, and a median survival time is approximately 12 months [1]. Although radiotherapy extends the survival for 6-8 months, it is not curative for the patient with GBM [2].

Recently it was reported that the invasiveness of glioblastoma cells were enhanced by photon irradiation [3-5]. Similar findings were also reported not only about GBM but also about other kind of cancers, including cancer of lung, liver, pancreas, kidney, and osteosarcoma in vitro and also in vivo [6-10]. On the other hand, our group previously showed that carbon ion
beam irradiation suppresses potentials of invasion, migration, and adhesion to extracellular matrix in fibrosarcoma cell line [11]. Carbon ion beam is well known as high-linear energy transfer (LET) radiation, and it has some unique characteristics compared with photon; high relative biological effect (RBE), oxygen or cell cycle independent damage, or high rate of double strand break [12, 13]. However, there are a lot of uncertain mechanisms of biological effects.

Hypoxic regions exist in almost all tumors because of its uncontrolled proliferation. In hypoxic condition, cancer cells become more aggressive because of acceleration of angiogenesis, glycolysis, and proliferation through the inhibition of hypoxia inducible factor 1-α (HIF1-α) degradation [14-16]. Moreover, recent reports suggest that hypoxia stimulates cancer cells’ invasiveness via activation of matrix metalloproteinase [17-20].

The purpose of this study is to investigate the effects of carbon ion beam irradiation on hypoxia-mediated metastatic potential of glioblastoma cell lines and clarify the mechanisms.

Materials and Methods

1. Cell culture and reagents
A172 and T98G, human glioblastoma cell lines, were maintained in RPMI1640 (Nissui Pharmaceutical Co. Ltd., Tokyo, Japan) containing 10% fetal bovine serum (FBS, Biowest, Nuaillé, France) and 0.3g/L L-glutamine at 37°C, 5% CO₂ in humidified camber. MG-132, cell permeable and selective proteasome inhibitor, was obtained from Biomol International (Plymouth Meeting, USA). As for western blotting, anti-mouse hypoxia inducible factor-1α (HIF-1α) monoclonal antibody and anti-mouse β-actin monoclonal antibody were obtained from Novus Biologicals (Littleton, USA) and Abcam (Cambridge, UK), respectively. Rho activation assay biochem kit and cell permeable Rho inhibitor were purchased from Cytoskeleton Inc. (Denver, USA).

2. Hypoxic condition
A hypoxic condition was maintained using an Anaeropack for Cell (Mitsubishi Gas Chemical Co., Tokyo, Japan). The Anaeropack for Cell contains sodium ascorbate as the principal ingredient, which absorbs oxygen and generates carbon dioxide by oxidative degradation. The cell culture flasks were placed into polyethylene-nylon bag with Anaeropack and Anaero-indicator (Mitsubishi Gas Chemical Co.). The bag was subsequently incubated in CO₂ incubator at 37°C for 5 hr. Then medium was changed and incubated in the incubator to reoxygenate hypoxic cells. Cells were irradiated 1-2 hr after medium change.

3. Irradiation
The cells were irradiated with 290 MeV/nucleon carbon ion beams at National Institute of Radiological Sciences (NIRS) in Japan. Cell culture flask was collocated at the center of 6 cm spread-out Bragg peak and was irradiated from horizontal direction. The irradiation system for carbon ion beam at Heavy Ion Medical Accelerator in Chiba (HIMAC) and the physical and biological characteristics of the beam has been described elsewhere [21-24]. For the X- irradiation, Cell culture flask was irradiated with 4MV X-ray, using linear accelerator (EXL-6SP, Varian, Palo Alto, USA). The dose rate was 100cGy/min. Serum free medium was added into the flask 1 hour before irradiation as build-up and the medium was immediately changed after irradiation.

4. Colony formation assay
Survival curves were obtained by means of standard colony formation assay. Irradiated cells were plated onto 60 mm-diameter plastic Petri-dishes, aiming for 30–60 colonies per dish. After 14days of incubation, cells were fixed with 10% formalin and stained with crystal violet. Colonies with >50 cells were scored as surviving colonies.

5. Immunoblotting
Lysates were generated by lysing with NP40 lysis buffer (50mM Tris-HCl, pH8.0, 120mM NaCl, 1mM EDTA, 0.2% NP40). Samples were prepared in sample buffer and heated to 95°C for 5min. Samples were run on 7.5–10% polyacrylamide gels and separated. The protein was transferred to Nitrocellulose (Whatman, Dassel, Germany) or PVDF membranes (Millipore, Bedford, USA), and the membrane was blocked
for 1hr in TBS-T (Tris buffer solution with 0.2% Triton X) containing 5% skim milk. The membrane was then incubated overnight with primary antibody at 4°C. Subsequently, the membrane was washed in three times with PBS-T (phosphate buffer saline with 0.2% Triton X) and incubated with anti-mouse IgG conjugated to horseradish peroxidase (GE healthcare Bio-Sciences, Little Chalfont, UK) for 1 hr. Membrane was then washed three times with PBS-T three times, and luminescence (GE Healthcare BioSciences) with X-ray film (Fuji Photo Film Ltd., Tokyo, Japan).

6. Matrigel invasion assay
Chemotaxis cell filters (Kurabo Industries Ltd, Osaka, Japan) with an 8 µm pore size were pre-coated with 10 µg/mL of Matrigel (Becton Dickinson Bioscience, Bedford, USA). Irradiated cells were trypsinized, washed twice with RPMI supplement with 0.1 % BSA. RPMI medium with 10% FBS was added to the bottom chambers of the wells, and cells (1 x 10^5) were added to the upper chambers. After incubation in 5% CO2 for 24 hr at 37°C, the cells were fixed with 10% formalin, stained with hematoxylin and eosin. The upper side filter was scraped off with a cotton swab and mounted on glass slides. The number of cells that had migrated to the lower side of filter was counted at x200 magnification in four independent fields.

7. Transwell motility assay
Chemotaxis was assessed using a 48-well microchemotaxis chamber (Neuro Probe, Gaithersburg, USA) and polycarbonate filter of 8-µm pores (Neuro Probe). Lower side of the filter was precoated with 10 µg/mL of collagen type I-C (Nitta Gelatin Inc., Osaka, Japan). The cells were trypsinized and resuspended in 0.1 % BSA, and then 2.5 x 10^5 cells were added to the upper chambers. Medium containing 10% FBS were placed into the bottom chamber. Cells were incubated on the membranes for 2 hr, fixed with 10% formalin, and stained with hematoxylin. And then the filter was placed on glass slides after the remaining cells on the upper side were scraped off with a cotton swab. The number of cells that had migrated to the lower side through the pores was scored at x200 magnification in four independent fields.

8. Affinity precipitation of Rho-GTP
Activation of RhoA was assessed, using Rho activation assay biochem kit (Cytoskeleton Inc.). Twenty-four hours after irradiation, cells were lysed in lysis buffer. Subsequently rhotekin-RBD beads was added and the samples were incubated at 4°C on a rotator for 1 hr. Samples were then centrifuged and washed with wash buffer. Sample buffer was added to bead samples, and they were boiled to 95°C for 5min.

9. Statistics
The results were expressed as mean values with standard deviations. The statistical significance was tested by means of Student’s t-test. A p-value of less than 0.05 was considered to be statistically significant.

Results
1. Calculation of relative biological effectiveness
To obtain biologically equivalent doses for each radiation quality, clonogenic survival curves were created using colony formation assay. The relative biological effectiveness value calculated by the dose for 1% survival of A172 cells was 2.22 for carbon ion beam relative to X-ray (Fig. 1). RBE of T98G cells is described as 2.00 [24].

![Fig. 1 Clonogenic survival curves after irradiation for A172 cells. Surviving fractions were plotted and fitted to surviving curves using the following linear-quadratic model: SF = exp(-αD-βD^2), where SF is the surviving fraction and D is the physical doses.](image-url)
Therefore, in subsequent assays, we applied half amount of physical doses of X-ray to doses of Carbon ion beam.

2. Verification of intracellular hypoxia

Oxygen level in the equipment for hypoxia was assessed with Anaero-indicator. In hypoxic condition \((O_2 < 0.1\%)\), the color of the reagents change from blue to pink. The condition of oxygen level was checked every time (data not shown). To verify whether hypoxic condition influenced cells, the expression of hypoxia inducible factor-1α (HIF-1α) was evaluated in protein level with immunoblotting (Fig 2). HIF-1α expression of cells incubated in hypoxia for 5 hr notably increased. Positive control was created by incubating with 25µM of MG-132 for 5 hr. The expression of HIF-1α distinctly reduced when the cells were reoxygenated and incubated for 1 hr after hypoxic incubation.

Fig. 2 Expression of HIF-1α (120kDa) in protein level was assessed by Western blotting analysis. β-actin (42kDa) is shown as loading control.

Fig. 3 Invasiveness of glioblastoma cells is examined by Matrigel invasion assay. As for A172 cells, the assay was carried out after (A) carbon beam irradiation or (B) X-irradiation. The invasiveness of T98G cells was also examined (C: carbon beam, D: X-ray). Normoxic cells; Hypoxic cells. Columns, mean; bars, ± SD. *, \(p < 0.05\) (Student’s t test, compared with untreated normoxic cell).
3. Effects of hypoxia and irradiation on cell invasion

To assess the effect of X-ray and carbon ion beam on cell invasive capability, Matrigel invasion assay was carried out. Invasiveness of A172 cells was up-regulated by hypoxia (Fig. 3A and B). As for X-ray, 0.5 Gy of X-irradiation enhanced the invasiveness more exceedingly (Fig 3A). On the other hand, carbon ion beam suppressed invasive potential enhanced by hypoxia (Fig.3B). There was no significant difference between control and hypoxic cells irradiated by 1 and 4 Gy of carbon ion beam. The similar results were obtained in T98 cells, and its enhancement by X-irradiation was more notable (Fig. 3C and D).

4. Effects of irradiation on cell migration

Migration is also fundamental component of tumor cell progression. We next focused on the effect of hypoxia and irradiation on motility using chemotaxis assay. Migration was also up-regulated by hypoxia (Fig. 4). As for the X-ray, migration of both normoxic and hypoxic

Fig. 4 Migratory potential of glioblastoma cells is assessed by transwell migration assay. As for A172 cells, the assay was carried out after (A) carbon beam or (B) X-ray irradiation. The migration of T98G cells was also tested (C: carbon beam, D: X-ray). Normoxic cells; Hypoxic cells. Columns, mean; bars, ± SD. *, p < 0.05 (Student’s t test, compared with untreated normoxic cell).
A172 cells was enhanced in dose dependent manner (Fig.4B). The function of T98G cells was increased by 0.5Gy of X-irradiation (Fig.4D). On the other hand, carbon particle irradiation suppressed the motility of both normoxic and hypoxic cells in dose dependent manner (Fig 4A, C).

5. Carbon ion beam suppresses RhoA activation
Both photon-irradiation and hypoxia are reported to enhance cell invasion in Rho signaling dependent manner [5, 17]. Small GTPases are involved in the motility and invasion of various carcinoma cell lines [25, 26]. To clarify the intracellular mechanisms responsible for the hypoxia-mediated invasion and radiation related changes of invasive potential, the effects of these stimuli on activation of RhoA were examined by GTP-Rho pull down assay (Fig.5). As for normoxic T98G cells, active RhoA was reduced by 1 and 4Gy of carbon ion beam irradiation, while significant difference was not indicated in hypoxic cells (Fig.5A). On the other hand, X-irradiation increased active RhoA of both normoxic and hypoxic cells in dose dependent manner (Fig.5B). Without irradiation, no difference was detected between normoxic and hypoxic cells.

6. Inhibition of RhoA activity down-regulates migration and invasion
To clarify whether inactivation of RhoA is involved in radiation-mediated effects on motility and invasiveness, the similar assays were performed using exoenzyme C3 Transferase, Rho inhibitor (Cytoskeleton Inc.). After incubation with C3 (2µg/mL) for 4 hr, Matrigel invasion

![Figure 5](image_url)

**Fig. 5** The effect of irradiation and hypoxia on the activation of RhoA in was assessed by pull down assay after cells were irradiated by (A) carbon beam and (B) X-ray. GTP-RhoA (28kDa) was precipitated using Rhotekin-GST conjugated beads, and then indicated by immunoblotting.
assay and transwell migration assay were carried out (Fig.6). Both assays demonstrated that Rho inhibitor suppresses cell invasion and migration in GBM cells. Therefore, it’s conceivable that carbon ion beam irradiation suppressed GBM cells’ invasion and migration via inactivation of RhoA signaling.

Discussion
Glioblastoma multiform (WHO Grade IV) is the most aggressive brain tumor. The standard treatment of GBM includes surgical resection with postoperative external beam radiotherapy. When surgical extirpation is not feasible, whole-brain irradiation is the primary treatment [27]. Fast neutrons, which are uncharged high-LET particles, have been used to treat malignant gliomas for many years. However, fast neutron radiotherapy has been discontinued because of unacceptable damage to the surrounding normal brain tissues [28]. Currently clinical trials using boron neutron capture therapy are ongoing [32-31].

High-LET charged particle radiation, such as carbon and neon, has been applied to various malignant tumors because of its great dose localizing property and other biological advantages. Previously our group showed that carbon particle irradiation suppresses metastatic potential of sarcoma cells in vitro and in vivo [11]. Carbon ion beam inhibited migratory potential, invasiveness, expression of β1 and αvβ3 integrin, and activation of matrix metalloproteinase-2 of fibrosarcoma cells, and implantation of osteosarcoma cells to lung of mice was also suppressed. The metastatic potential of human lung adenocarcinoma cell line (A549) was also suppressed by carbon ion beam (data not shown). Our group also showed that carbon ion beam inhibits angiogenesis, fundamental component for progression of tumor [32]. These results suggest that charged particle has distinctly different effects on biological activity from photon irradiation.

In this study, hypoxic condition promoted migration and invasion of the cells. Corresponding to some previous reports [17-20]. Hypoxic region exists in malignant tumors because of unregulated proliferation and insufficient angiogenesis, and the degradation of hypoxia inducible factor-1α (HIF-1α) is inhibited in hypoxic part of tumor. HIF-1α is well known as an important mediator of the hypoxic response of tumor cells, and it controls the up-regulation of a number of factors vital for solid tumor expansion, including angiogenic factors, erythropoietin, and glucose transporter-1 [33, 34].

X-irradiation is also accelerated invasive
potential of glioblastoma cells. Zhai et al. suggested that photon irradiation stimulates GBM cells’ aggressiveness through Rho signaling activation [5]. Interestingly, hypoxia-mediated invasion is also reported to be involved in Rho signaling [16]. The Rho GTPases, including Rho, Rac, and Cdc42, work as molecular switches in cell morphogenesis by inducing specific types of actin cytoskeleton and by locally regulating microtubules dynamics [35]. GTP-bound RhoA (active form) acts by regulating the activity of effector proteins, including Rho-associated kinase (ROCK) and mammalian homologue of the Drosophila gene Diaphanous 1 (mDia1). ROCK is involved in focal adhesion and actin stress fiber, and contributes to the Ras-transformed phenotype [36, 37]. Furthermore, Itoh et al. demonstrated that ROCK is an essential part for cell invasion [38]. mDia1 regulates microtubules dynamics and cell polarity [39]. Rho and ROCK are reported to activate Rac though mDia, and Rac also regulates cell invasion via phosphatidylinositol 3-kinase (PI3K) [40-42]. Therefore, Rho signaling plays a vital role for cell invasion. In current study, carbon ion beam inhibited invasion and activation Rho signaling. Moreover, invasive potentials enhanced by hypoxia were also suppressed by carbon ion beam. While the influence to hypoxic cancer cells by X-ray irradiation was reported, the effect by carbon ion beam was elucidated in this study for the first time. Conceivably inhibition of Rho activation will be the decisive reason of carbon ion beam-mediated suppression of invasiveness.

Cell motility is associated with cell adhesion, and Rho signaling also regulates this function. Rho-mDia1 pathway modulates polarization and adhesion turnover during cell movement [43]. Current study showed that migratory potential of glioblastoma cells was also enhanced by hypoxia and X-irradiation respectively, and carbon ion beam suppressed this capability even though the function was promoted in hypoxic condition. Therefore, inactivation of RhoA induced by carbon ion irradiation might be involved in suppression of cell migration.

Our group previously showed that carbon ion beam decreased the expression of β1 and αvβ3 integrin in human fibrosarcoma cells [11]. The integrin family of adhesion molecules is extracellular matrix receptors, and integrin-mediated adhesion to extracellular matrix triggers intracellular signaling pathways to modulate cell proliferation, shape, and migration [44, 45]. Clark et al. suggested that Rho protein regulates cell spreading and focal adhesion through activation of FAK, and phosphorylation of paxillin [46]. They are the major components of integrin-modulated signaling pathway. Thus carbon ion beam irradiation might suppress the function of integrin via inhibition of its expression and signaling.

However RhoA was not activated in hypoxic condition in this study. Zhai et al. reported that radiation-mediated invasion of GBM is involved in insulin like growth factor receptor, epidermal growth factor receptor, PI3K and Rho, but not in serin/threonine kinase Akt (protein kinase B) signaling [5]. On the other hand, Cheng et al. suggested that radiation enhances invasion of hepatocellular carcinoma through PI3K, Akt, and nuclear factor-κB (NF-κB) signaling [47]. Therefore, radiation-related stimulation pathway of cells might be cell type dependent. The result of RhoA pull down assay and Rho inhibition assay using T98G cells demonstrate that carbon ion beam irradiation suppresses invasion and migration via inactivation of Rho signaling. Our previous study indicated that carbon ion beam irradiation to A549 cells reduced the expression of anillin in mRNA level (data not shown). Anillin, actin binding protein, is reported to form a complex with Rho [48]. Thus it is conceivable that carbon particle suppressed Rho activation through this pathway.

Recently charged particle irradiation is reported to be used for the treatment of patients with malignant gliomas. Mizoe et al. suggested the results of a Phase I/II clinical trial for patients with malignant gliomas, treated with combined X-ray, chemotherapy using nimustine hydrochloride (ANCU), and carbon ion radiotherapy [49]. The results showed the effectiveness of higher carbon ion dose for better local control and median progression free survival of GBM. A similar result was reported by Castro et al. using neon ion radiotherapy [50]. Heavy ion beam obviously have great advantages compared with
X-ray, and our current study may contribute to the clarification of the mechanism. Moreover, further detailed clarification of this mechanism might be able to suppress metastasis by inhibiting the invasion-related pathway, even if X-irradiation therapy is applied.

In conclusion, carbon particle irradiation suppressed migration and invasion of glioblastoma via Rho signaling inactivation. Furthermore, carbon ion beam also suppressed the invasive potential enhanced by hypoxia.

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References


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