

Intracellular reactions affecting 2-amino-4-([¹¹C]methylthio)butyric acid ([¹¹C]methionine) response to carbon ion radiotherapy in C10 glioma cells

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Abstract

Purpose: The response of 2-amino-4-([¹⁴C]methylthio)butyric acid ([¹⁴C]Met) uptake and [¹²⁵I]3-iodo-α-methyl-L-tyrosine ([¹²⁵I]IMT) uptake to radiotherapy of C10 glioma cells was compared to elucidate the intracellular reactions that affect the response of 2-amino-4-([¹¹C]methylthio)butyric acid ([¹¹C]Met) uptake to radiotherapy.

Methods: After irradiation of cultured (3 Gy) or xenografted C10 glioma cells (25 Gy) using a carbon ion beam, the accumulation of [¹⁴C]Met and [¹²⁵I]IMT in the tumors was investigated. The radiometabolites in xenografted tumors after radiotherapy were analyzed by size-exclusion HPLC.

Results: [¹⁴C]Met provided earlier responses to the carbon ion beam irradiation than [¹²⁵I]IMT in both cultured and xenografted tumors. While [¹²⁵I]IMT remained intact in xenografted tumor before and after irradiation, the radioactivity derived from [¹⁴C]Met was observed both in high molecular fractions and intact fractions, and the former decreased after irradiation.

Conclusion: The earlier response of [¹¹C]Met uptake to tumor radiotherapy could be attributable to the decline in the intracellular energy-dependent reactions of tumors due to radiotherapy.

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Keywords: Radiotherapy; Carbon-ion; ¹¹C-methionine; Cellular activity

1. Introduction

Carbon ion radiotherapy started at the National Institute of Radiological Sciences (NIRS, Japan) using Heavy Ion Medical Accelerator in Chiba (HIMAC) synchrotron accelerator in 1994. So far, HIMAC at NIRS has treated over 3000 patients suffering from malignant disease [1–3]. Although a carbon ion beam has higher relative biological effectiveness and smaller differences in sensitivity between cell lines, the responses of tumor cells after irradiation using a carbon ion beam still differed between cells [4]. This calls for a precise and reliable method for estimating the

therapeutic efficacy of carbon ion beams at an early stage of the treatment.

In general, morphological diagnosis, such as X-ray computed tomography (CT) and magnetic resonance imaging (MRI), are used to assess the response of cancers to therapy [5]. However, morphological diagnosis is affected by the differentiation of viable tumor cells from necrosis/scar tissue. Moreover, it is necessary to wait for a long period after radiotherapy before an accurate evaluation can be made [6,7]. On the other hand, functional imaging with positron emission tomography (PET) using radiopharmaceuticals such as 2-[¹⁸F]-fluoro-2-deoxy-D-glucose ([¹⁸F]FDG) or 2-amino-4-([¹¹C]methylthio)butyric acid ([¹¹C]Met, Fig. 1A) can elucidate biochemical and physiological changes in tumor cells before morphological changes occur in the tumor

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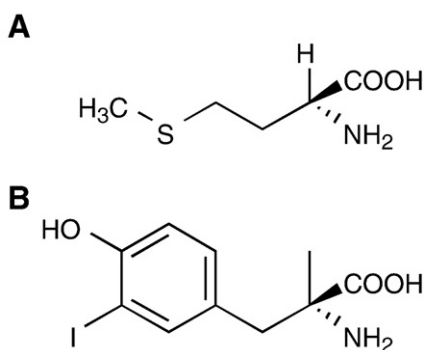


Fig. 1. Structures of Met (A) and IMT (B).

[8,9]. This imaging technique has been applied to estimate tumor responses to radiotherapy [7,10–13].

It is well known that irradiation induces an attraction and activation of inflammatory cells such as macrophages [13,14]. These inflammatory cells lead to a prominent increase in some radiopharmaceuticals such as [^{18}F]FDG comparable with that in tumor cells [13,14]. The radiopharmaceuticals that accumulated in the inflammation induced by irradiation made it difficult to predict the efficacy of tumor radiotherapy [13,14]. The lower accumulation of [^{11}C]Met at sites of irradiation compared with [^{18}F]FDG renders [^{11}C]Met as the radiopharmaceutical of choice for this purpose [13,14]. However, because cyclotron facilities are required to produce and use [^{11}C]Met, due to its short half-life (20 min), the development of ^{18}F ($T_{1/2}$: 110 min) or ^{123}I ($T_{1/2}$: 13 h)-labeled radiopharmaceuticals that afford diagnostic information similar to that of [^{11}C]Met is highly desirable.

The accumulation of [^{11}C]Met in tumors is mediated by several cellular reactions, such as amino acid transport across the membrane, intracellular protein synthesis and trans-methylation [9]. However, it remains uncertain which cellular reactions are directly correlated with the reduction in [^{11}C]Met accumulation after radiotherapy. The answer to this question paves the way for designing ^{18}F - or ^{123}I -labeled radiopharmaceuticals that afford diagnostic information comparable to [^{11}C]Met. The uptake of [^{11}C]Met by tumors is mediated predominantly by energy independent amino acid transport system L [15,16], whereas protein synthesis and trans-methylation are energy dependent reactions [17]. The artificial amino acid [^{123}I]3-iodo- α -methyl-L-tyrosine ([^{123}I]IMT, Fig. 1B) is also transported to tumor cells by amino acid transport system L [18–21], whereas [^{123}I]IMT is not involved in protein synthesis [22–24], indicating that the uptake of [^{123}I]IMT into tumors only reflects the activity of amino acid transport system L. This suggested that the comparative tumor accumulation of [^{11}C]Met and [^{123}I]IMT could provide insights for understanding the cellular reactions correlated with the response of tumors to [^{11}C]Met accumulation after radiotherapy.

In the present study, tumor accumulation and intracellular metabolism of [^{14}C]Met and [^{125}I]IMT were com-

pared after irradiation of cultured C10 glioma cells or the same cells xenografted into nude mice using a carbon ion beam. The critical cellular reactions responsible for the response of tumors to [^{11}C]Met accumulation after radiotherapy are discussed.

2. Materials and methods

2.1. General

2-Amino-4-([^{14}C]methylthio)butyric acid ([^{14}C]Met) was purchased from GE Healthcare Bioscience (Tokyo, Japan). Size-exclusion high-performance liquid chromatography (SE-HPLC) was performed using a Cosmosil 5Diol-300 column (7.5×600 mm, Nacalai Tesque, Kyoto, Japan) eluted with 0.1 M phosphate buffer (pH 6.8) at a flow rate of 1.0 ml/min. Each eluent was collected with a fraction collector (RadiFlac, GE Healthcare Bioscience) at 1-min intervals, and the radioactivity counts in each fraction (1 ml) were determined with an auto well gamma counter (ARC-380M, Aloka, Tokyo, Japan). ^{14}C -Radioactivity was measured with a liquid scintillation counter (LSC-5100, Aloka). [^{125}I]IMT was prepared according to the procedure described previously [24]. The C10 glioma cell line was provided from the National Institute of Radiological Sciences (NIRS, Chiba, Japan). Other reagents were of reagent grade and used without further purification.

2.2. Cell culture

C10 Glioma cells were grown as a monolayer in McCoy's 5A medium (Invitrogen, Tokyo, Japan) supplemented with 10% fetal bovine serum (SAFC Biosciences, Kansas City, MO, USA), penicillin (100 U/ml), and streptomycin (100 $\mu\text{g}/\text{ml}$) at 37°C in an atmosphere of 5% CO_2 . Before the cell uptake experiments, C10 glioma cells were washed twice with phosphate-buffered saline (PBS) and trypsinized using a solution of 0.05% trypsin in PBS. After the cells were resuspended in McCoy's 5A medium, the cell number was counted by a Coulter counter (Beckman Coulter KK, Tokyo, Japan).

2.3. Irradiation

Carbon ion beams were accelerated up to 290 MeV/U with the Heavy Ion Medical Accelerator in Chiba (HIMAC) synchrotron at NIRS. Modulator films were used to perform a LET value of 60 keV/ μm with a spread-out Bragg peak of 6-cm width. The sample was irradiated at room temperature.

2.4. In vitro experiments

Cell uptake experiments were performed according to the procedure of Samnick et al. [25] with slight modifications. The cell suspension was transferred to 2-ml Eppendorf tubes at a concentration of 7×10^5 cells. After the cells were centrifuged for 5 min at $300 \times g$, the resulting supernatant was removed, and the pellet was washed twice with 2 ml of

HEPES buffer (pH 7.4, 12 mM HEPES, 137 mM NaCl, 2.7 mM KCl, 1.0 mM MgCl₂, and 5.6 mM D-glucose). For the Na⁺-free uptake study, choline-Cl replaced NaCl. The cells were resuspended in HEPES buffer (500 μl) and preincubated for 15 min at 37°C to equilibrate the cells. Aliquots of 20 μl ([¹²⁵I]IMT: 11.1 kBq, 0.14 pmol, [¹⁴C]Met: 3.7 kBq, 1.6 nmol) were added to the cells in the presence or absence of an inhibitor for amino acid transporter system A or L [5 mM alpha-(methylamino) isobutyric acid (MeAIB) or 5 mM 2-amino-2-norbornane carboxylic acid (BCH), respectively], and the cells were incubated at 37°C for 3 min. After terminating the tracer uptake reaction using 1 ml of ice-cold PBS and a subsequent 2-min incubation in an ice bath, the cells were centrifuged for 2 min at 300×g. The resulting supernatant was removed, and the pellet was washed three times with ice-cold PBS (1 ml). ¹²⁵I-Radioactivity counts in the cells were measured with a gamma counter, while ¹⁴C-radioactivity levels were determined with a liquid scintillation counter (LSC-6000, Aloka) after cells were dissolved in tissue solubilizer (0.5 ml, SOLUENE-350, Packard Japan, Tokyo, Japan) and scintillator (5 ml, HYNIC-FLUOR, Packard Japan). The data were expressed as uptake per million cells. Each assay was performed in triplicate.

2.5. Cell uptake experiments after irradiation

The cells (5.6×10⁵ cells) were incubated in T-25 flasks (Nalge Nunc, Tokyo, Japan) for 4 days. Three to four flasks were used for each assay. The flasks were irradiated by a carbon ion beam (3 Gy) at room temperature. The cell survival rate after carbon ion beam irradiation (3 Gy) was about 30% of nonirradiated cells (data not shown). After irradiation, the medium was exchanged for a fresh medium. The cellular uptake of [¹²⁵I]IMT (11.1 kBq, 5 nmol) or [¹⁴C]Met (3.7 kBq, 5 nmol) by C10 glioma cells was investigated from 1 to 4 days after irradiation in accordance with the above procedure. The data were expressed as uptake per million cells. Each assay was performed in triplicate.

2.6. In vivo experiments

Animal studies were conducted in accordance with the institutional guidelines and were approved by the Chiba University Animal Care Committee. Four to five mice for each point were used. C10 Glioma cells (1×10⁶ cells) were transplanted subcutaneously into the left hind legs of nude mice 7 days before irradiation. Under pentobarbital anesthesia (20 mg/kg), the mice were immobilized on a Lucite plate to place their left hind legs in the irradiation field. The tumor-containing left hind legs received carbon ion beam irradiation (25 Gy). No tumor regrowth was observed for one month after carbon ion radiotherapy (25 Gy) (data not shown). Tumor diameters were measured by external measurement in three dimensions using a caliper, and tumor volumes (*v*) were determined using the formula $v = a \times b \times c$ (where *a*, *b* and *c* represent the tumor diameter). [¹²⁵I]IMT (11.1 kBq, 0.14 pmol) and [¹⁴C]Met (11.1 kBq,

5 nmol) dissolved in 100 μl of saline were simultaneously administered intravenously before sacrifice by decapitation after 1 h. The difference in the specific activities of these radiopharmaceuticals could be ignored because the methionine concentration in the serum of the mice is high (ca. 300 μM) [26]. Tissues of interest were removed and weighed, and the ¹²⁵I-radioactivity counts were determined using an auto well gamma counter (Aloka). The tissue was then dissolved in soluene-350 (0.5 ml, Packard Japan) and Hionic-Fluor (4 ml, Packard Japan). The ¹⁴C-radioactivity counts were measured with a liquid scintillation counter (Aloka). ¹⁴C-Radioactivity counts were determined by subtracting the ¹²⁵I-radioactivity counts from the total count. On the other hand, to evaluate the accumulation of [¹²⁵I]IMT and [¹⁴C]Met at the irradiated site, the left hind legs (muscle) without tumors were also irradiated using the carbon ion beam (25 Gy) and investigated by the same procedure as above.

2.7. Metabolic studies in tumor

C10 Glioma cells (1×10⁶ cells) were transplanted subcutaneously into the left hind legs of nude mice 7 days before the irradiation. The tumor-containing left hind legs were irradiated by the carbon ion beam (25 Gy) as described above. Two days after irradiation, [¹²⁵I]IMT (740 kBq, 5 pmol) and [¹⁴C]Met (740 kBq, 0.17 μmol) dissolved in 100

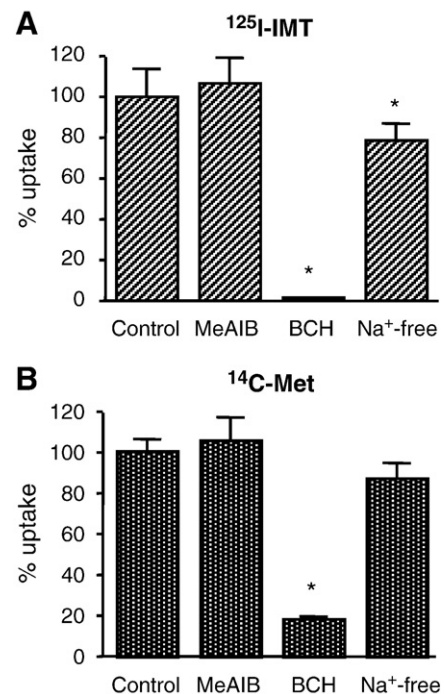


Fig. 2. The uptake of [¹²⁵I]IMT (A) and [¹⁴C]Met (B) by C10 glioma cells. Values are mean±S.D. MeAIB, an inhibitor of amino acid transporter system A, showed no significant inhibitory effect on [¹²⁵I]IMT or [¹⁴C]Met uptake. An inhibitor of amino acid transporter system L, BCH, significantly reduced the uptake of both [¹²⁵I]IMT (98%) and [¹⁴C]Met (82%).

μl of saline were simultaneously administered intravenously to mice before being sacrificed by decapitation after 1 h. Tumor tissues were removed and weighed. Tumor samples (0.5 g) were placed in a test tube and subjected to three cycles of freezing (dry ice-methanol bath) and thawing. After the addition of 5 volumes of 0.1 M Tris-buffer (pH 6.5) containing 0.15 M NaCl, 0.02% sodium azide, 1 trypsin inhibitor unit/ml aprotinin, 2 mM benzamide-HCl, 2 mM iodoacetamide, 1 mM phenylmethanesulfonyl fluoride, 5 mM diisopropyl fluorophosphates and 35 mM β -octylglucoside, the samples were homogenized using a Polytron

homogenizer (PT10-35, Kinematica, Littau, Switzerland) at full speed with three consecutive 30-s bursts with incubation on ice before centrifugation at $48000\times g$ for 20 min at 4°C (Optima MAX, Beckman Coulter KK). The supernatant was separated from the pellet. The tumor supernatant was analyzed by SE-HPLC after filtration through a polycarbonate membrane with a pore diameter of $0.45\ \mu\text{m}$ (Nacalai Tesque). Two mice for each point were used, and the data were expressed as the average of the two experiments.

2.8. Statistical evaluation

Unpaired Student's *t* test was used for the experiments. Results were considered statistically significant at $P < .05$.

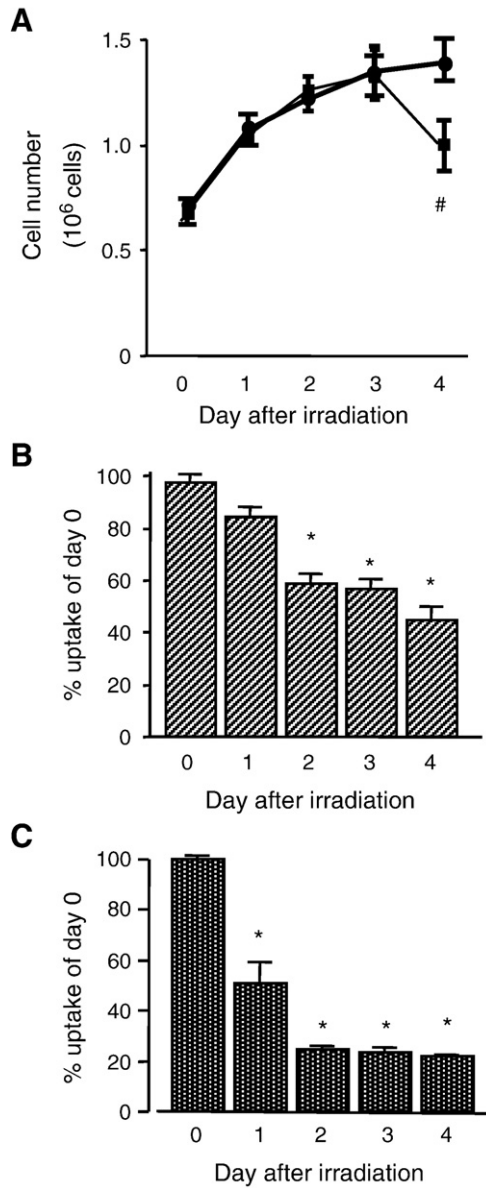


Fig. 3. Time course of changes in the cell numbers [control (circle), irradiation (square)] per flask (A), $[^{125}\text{I}]\text{IMT}$ uptake (B) and $[^{14}\text{C}]\text{Met}$ uptake (C) by C10 glioma cells after irradiation using the carbon ion beam (3 Gy). Values are mean \pm S.D. ($n=3$). Significant differences were determined by unpaired Student's *t* test. [#] $P < .05$ vs. control; * $P < .05$ vs. Day 0.

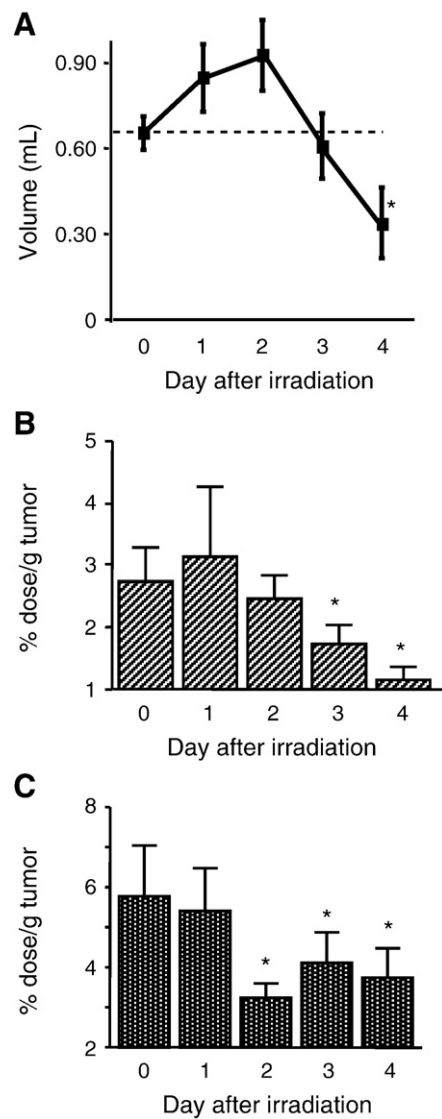


Fig. 4. Time course of changes in tumor volume (A), $[^{125}\text{I}]\text{IMT}$ uptake (B) and $[^{14}\text{C}]\text{Met}$ uptake (C) in xenografted C10 glioma-bearing nude mice after irradiation using the carbon ion beam (25 Gy). Values are mean \pm S.D. ($n=4-5$). Significant differences were determined by unpaired Student's *t* test. * $P < .05$ vs. Day 0.

3. Results

3.1. In vitro experiments

The tumor uptake of [^{125}I]IMT was not reduced in the presence of MeAIB, an inhibitor of amino acid transport system A, but was almost completely (98%) inhibited in the presence of BCH, an inhibitor of amino acid transporter system L (Fig. 2). In Na^+ -free buffer, [^{125}I]IMT uptake was reduced to 21% of the control. [^{14}C]Met uptake was not inhibited by MeAIB but was reduced by BCH to 82% of the control. In Na^+ -free buffer, [^{14}C]Met uptake was reduced to 12% (Fig. 2).

The time course of changes in cell number after irradiation using the carbon ion beam (3 Gy) is shown in Fig. 3. The cell number increased until 3 days after irradiation and then decreased with time. The uptake of [^{125}I]IMT was reduced to 87% and 60% of Day 0 at 1 and 2 days post-irradiation, respectively. The [^{125}I]IMT uptake decreased slightly with time. The uptake of [^{14}C]Met was reduced to 50% and 25% of Day 0 at 1 and 2 days post-irradiation. Thereafter, the uptake of [^{14}C]Met remained unchanged.

3.2. In vivo experiments

Fig. 4 shows the time course of tumor volume changes after irradiation using the carbon ion beam (25 Gy). The tumor volumes continued to increase until 2 days after irradiation and then began to shrink. On Day 4, the volumes became significantly smaller (39%) compared to those at Day 0. On Day 3, the uptake of [^{125}I]IMT was significantly decreased compared to that on Day 0 (65%, $P < .05$). A significant reduction in [^{14}C]Met uptake (41%, $P < .05$) was observed from Day 2 (Fig. 4C). No tumor regrowth was observed for one month (data not shown).

Fig. 5 depicts the uptakes of [^{125}I]IMT and [^{14}C]Met by irradiated or nonirradiated muscle for 5 days after irradiation using the carbon ion beam (25 Gy). No significant

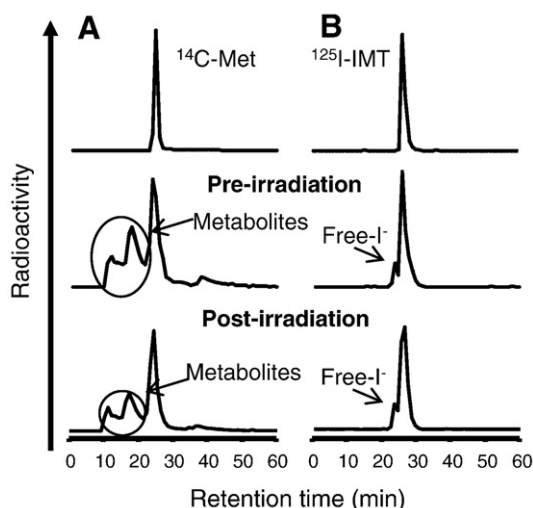


Fig. 6. SE-HPLC radiochromatograms of [^{14}C]Met or [^{125}I]IMT (top) and of tumor extracts 60 min after the injection of [^{14}C]Met (A) and [^{125}I]IMT (B) before (middle) or after (bottom) carbon ion beam irradiation. Under these conditions, Met, IMT and free iodide had retention times of 25, 26, and 24 min, respectively.

differences were observed in [^{125}I]IMT and [^{14}C]Met accumulation between irradiated and non-irradiated muscle.

Fig. 6 shows the SE-HPLC elution profiles of radioactivity from tumor extracts after injection of [^{125}I]IMT or [^{14}C]Met into mice before and 2 days after irradiation using the carbon ion beam (25 Gy). The extracts of tumor homogenates were obtained with radiochemical efficiencies of 85%. More than 90% of the radioactivity was detected as intact [^{125}I]IMT in irradiated and nonirradiated tumors. The rest of the radioactivity was observed in fractions similar to those of iodide. On the other hand, two additional radioactivity peaks were observed at earlier retention times after injection of [^{14}C]Met in both irradiated and nonirradiated tumors. While the high molecular weight fractions represented 51% of the total radioactivity in non-irradiated

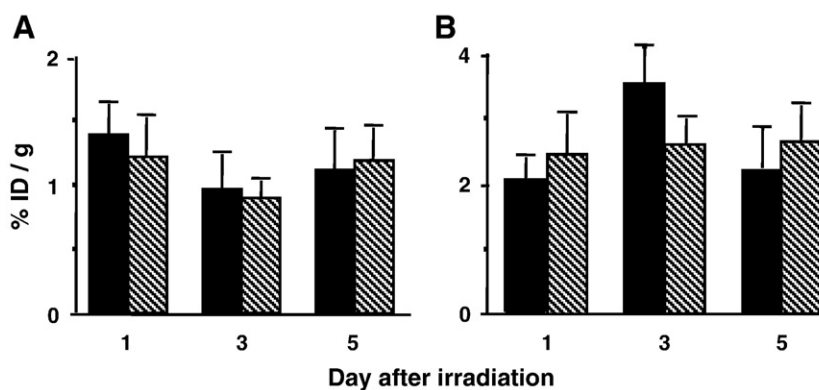


Fig. 5. Uptake of [^{125}I]IMT (A) and [^{14}C]Met (B) at irradiation sites (solid bar) after irradiation using the carbon ion beam (25 Gy) and at non-irradiated site (hatched bar). Values are mean \pm S.D.

tumors, the radioactivity of the fractions decreased to 43% of the total radioactivity in irradiated tumors.

4. Discussion

The accumulation of [^{14}C]Met in carbon ion beam irradiated tumor cells was reduced before morphological changes (cell number and tumor volumes) of the tumor became apparent (Figs. 3 and 4). These results were in good agreement with the clinical studies reported previously [13,14]. The present studies also confirmed that the uptake of both [^{14}C]Met and [^{125}I]IMT into tumor cells was mediated by amino acid transport system L (Fig. 2). The accumulation of both [^{14}C]Met and [^{125}I]IMT at sites of irradiation was not observed (Fig. 5). These findings supported the validity of this study design that the comparison of [^{14}C]Met accumulation and metabolism with [^{125}I]IMT in irradiated or nonirradiated tumor cells could provide an insight for assessing the critical cellular reactions directly correlated with [^{11}C]Met accumulation in tumors.

The reduction of both [^{14}C]Met and [^{125}I]IMT occurred in advance of the reduction of cell number and tumor volume (Figs. 3 and 4). This indicated that a dysfunction in amino acid transport system L could be involved in the response of tumor cells to [^{14}C]Met uptake. However, while the reduction in [^{14}C]Met accumulation in irradiated tumor cells occurred 2 days after irradiation, [^{125}I]IMT showed a significant decrease in tumor accumulation 3 days after irradiation (Figs. 3 and 4). This indicated that cellular reactions other than amino acid transport system L could also be involved in the tumor response to [^{14}C]Met accumulation.

To further examine the cellular reactions, the metabolism of [^{14}C]Met and [^{125}I]IMT in tumor cells was compared by SE-HPLC before and 2 days after irradiation, because at 2 days after irradiation of tumors using the carbon ion beam, the tumor uptake of [^{14}C]Met decreased, but that of [^{125}I]IMT was not changed (Fig. 4). The majority of [^{125}I]IMT remained intact in the tumor, and no changes in HPLC radioactivity profiles were observed between irradiated and non-irradiated tumors (Fig. 6). Contrary to [^{125}I]IMT, ^{14}C -radioactivity was detected in both high-molecular-weight fractions and the intact [^{14}C]Met fraction in tumors. In addition, a decrease in the high molecular weight fraction was observed in irradiated tumors (Fig. 6). A significant reduction in the tumor accumulation of [^{14}C]Met was observed at this postirradiation time (Fig. 4). It is well known that methionine is incorporated into protein synthesis and that the methyl group of methionine is transferred to RNA, DNA, and polyamines in the cells [9,27]. These intracellular processes are also known to proceed in an energy-dependent manner [17]. On the other hand, it was reported that, in mice given cycloheximide to inhibit protein synthesis, the tumor uptake of [^{14}C]leucine decreased more than that of [^3H]Met in tumor cells, although they were both transported by amino acid transporter system L [9]. Because

the uptake of Met reflects not only protein synthesis but also other metabolic pathways, such as transmethylation, the uptake of Leu reflects protein synthesis. Thus, the findings in this study implied that energy-dependent intracellular reactions such as protein synthesis and trans-methylation could constitute the key reactions that affect the changes in [^{11}C]Met accumulation in carbon ion beam-irradiated tumors. Although the intracellular reactions are inherent to [^{11}C]Met, radiopharmaceuticals that reflect the activities of energy-dependent intracellular reactions may be useful for predicting the therapeutic efficacies.

In conclusion, the present study assessed the cellular reactions directly correlated with the changes in [^{11}C]Met accumulation of the tumor cells. While the dysfunction of amino acid transport system L was involved, energy-dependent intracellular activities could constitute the key reactions that affect the radioactivity levels in the tumor cells after injection of [^{11}C]Met. These results also suggest that radiopharmaceuticals that reflect the activities of energy-dependent intracellular reactions may be useful to predict tumor responses to carbon ion radiotherapy.

Acknowledgments

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