

# Particle Irradiation Suppresses Metastatic Potential of Cancer Cells

Toshiyuki Ogata,<sup>1,3</sup> Teruki Teshima,<sup>1</sup> Kazufumi Kagawa,<sup>3</sup> Yoshio Hishikawa,<sup>3</sup> Yutaka Takahashi,<sup>1</sup> Atsuko Kawaguchi,<sup>1</sup> Yuko Suzumoto,<sup>1</sup> Kumie Nojima,<sup>4</sup> Yoshiya Furusawa,<sup>5</sup> and Nariaki Matsuura<sup>2</sup>

Departments of <sup>1</sup>Medical Physics & Engineering and <sup>2</sup>Molecular Pathology, Osaka University Graduate School of Medicine, Suita, Osaka, Japan; <sup>3</sup>Department of Radiology, Hyogo Ion Beam Medical Center, Ibo-gun, Hyogo, Japan; and Laboratories of <sup>4</sup>International Space Radiation and <sup>5</sup>Heavy-ion Radiobiology for Therapy, National Institute of Radiological Sciences, Inage-ku, Chiba, Japan

## Abstract

**Particle radiotherapy such as proton and carbon ion has been producing promising clinical results worldwide. The purpose of this study was to compare metastatic capabilities of malignant tumor cells after irradiation with photon, proton, and carbon ion beams to clarify their ion beam-specific biological effects. We examined the biological properties of highly aggressive HT1080 human fibrosarcoma cells to assess their metastatic processes in terms of cell adhesion capability to extracellular matrix, expression of integrins, cell migration, cell invasive capability, and matrix metalloproteinase-2 activity *in vitro*. We then assessed the metastatic capabilities of LM8 mouse osteosarcoma irradiated with carbon ion or photon beam in the syngeneic mice. Both proton and carbon ion irradiation decreased cell migration and invasion in a dose-dependent manner and strongly inhibited matrix metalloproteinase-2 activity. On the other hand, lower X-ray irradiation promoted cell migration and invasion concomitant with up-regulation of  $\alpha$ V $\beta$ 3 integrin. For cancer cells treated with carbon ion irradiation, the number of pulmonary metastasis was decreased significantly *in vivo*. These findings suggest that particle irradiation suppresses metastatic potential even at lower dose, whereas photon irradiation promotes cell migration and invasive capabilities at lower dose level, and provide preclinical evidence that ion beam radiotherapy may be superior to conventional photon beam therapy in possible preventive effects on metastases of irradiated malignant tumor cells.** (Cancer Res 2005; 65(1): 113-20)

## Introduction

Metastasis, the biggest threat to survival for patients with solid tumors, is the spread of tumor cells from the original growth to the other sites in the body. Metastatic processes of malignant tumor cells generally consist of (i) detachment of cells from the primary tumor, (ii) migration to extracellular matrices, (iii) degradation of basement membrane, (iv) invasion into blood vessels, (v) circulation in blood flow, (vi) escape to extravascular matrices, and (vii) implantation to target organs. These processes are based upon a number of biological characteristics associated with various molecular changes involving proteinases, adhesion molecules, and cell motility factors.

The integrin family of adhesion molecules is extracellular matrix receptors consisting of  $\alpha$  and  $\beta$  chains that form various

heterodimers with distinct cellular and adhesive characteristics. Integrin-mediated adhesion to extracellular matrix triggers intracellular signaling pathways to modulate cell proliferation, shape, migration, invasion, and survival (1, 2). The  $\beta$ 1 integrin subfamily consists of a receptor subunit associated with several  $\alpha$  subunits resulting in a broad spectrum of receptors for a variety of potential ligands (3, 4). The vitronectin receptor,  $\alpha$ V $\beta$ 3 integrin, also seems to be associated with increased invasiveness (5, 6).

Matrix metalloproteinases (MMP) constitute a family of Zn<sup>2+</sup>-dependent enzymes essential for extracellular matrix turnover under normal and pathologic conditions. Especially MMP-2 can degrade type IV collagen, one of the major components of the basement membrane, resulting in the promotion of tumor invasion and metastasis (7). One of the mechanisms of this process is that MMP-2 directly binds to  $\alpha$ V $\beta$ 3 integrin and thus localizes in a proteolytically active form on the surface of invasive cells (8).

In the clinic, ionizing radiation has been established as a highly effective modality used in the local control of tumor growth. However, several authors have reported that photon beam irradiation enhanced metastatic processes of malignant tumor cells at sublethal dose (9–13). New types of radiation sources, particle beams such as proton and carbon ion, may be expected to be a new modality of cancer treatment. Particle therapy has the advantage, in theory, over conventional photon beam that the tumor can be targeted with extreme precision, without damage to normal surrounding tissue, either superficial or deep, thereby allowing for an extraordinary escalation of dosage to the tumor. Carbon ion with high linear energy transfer has been shown more effective than photon and proton for cell-killing effect (14–16). Only a few studies have been conducted of the effects of particle beams on functioning of cells with metastatic potential. Our group was the first to report that carbon beam irradiation inhibited *in vitro* angiogenesis even at sublethal dose (17).

We show metastatic potential after irradiation with photon, proton, and carbon ion beams to elucidate particle-specific biological effects. Here, we report that particle irradiation suppresses metastatic potential, whereas photon irradiation promoted cell migration and invasive capabilities at lower dose level.

## Materials and Methods

**Cell Culture and Reagents.** Highly aggressive HT1080 human fibrosarcoma (American Type Culture Collection, Rockville, MD) and LM8 mouse osteosarcoma (18), a highly metastatic Dunn cell subline which was kindly given by Dr. Yoshikawa (Osaka University, Osaka, Japan), were maintained in DMEM medium (Nihonseiyaku, Tokyo, Japan) with 10% fetal bovine serum and penicillin/streptomycin (Invitrogen, Carlsbad, CA) at 37°C in a humidified atmosphere of 10% CO<sub>2</sub> and 90% air. The MMP inhibitor GM6001 was purchased from Chemicon (Temecula, CA).

**Requests for reprints:** Teruki Teshima, Departments of Medical Physics & Engineering, Osaka University Graduate School of Medicine, 1-7 Yamada-oka, Suita, Osaka 565-0871, Japan. Phone: 166-879-2570; Fax: 166-879-2570; E-mail: teshima@sahs.med.osaka-u.ac.jp.

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**Irradiation.** Cell irradiation with 190 MeV/nucleon proton beams was done at the Hyogo Ion Beam Medical Center in Japan. Cells were irradiated at the center of Bragg peaks modulated to 6-cm widths. The irradiation system and biophysical characteristics of proton beams have been detailed elsewhere (19).

For carbon ion irradiation, cells were treated with 290 MeV/nucleon carbon ion beams at 6-cm spread-out Bragg peak center from the Heavy Ion Medical Accelerator in Chiba at the National Institute of Radiological Sciences in Japan. The irradiation system for carbon ion at Heavy Ion Medical Accelerator in Chiba and the physical characteristics of the beam have been described elsewhere (20, 21).

For photon irradiation, 4 MV X-ray from the linear accelerator at Osaka University Graduate School of Medicine was used with a delivered dose rate of  $\sim 1.8$  Gy/min.

**Colony Formation Assay.** Survival curves were obtained by means of standard colony formation assay. Irradiated cells were plated onto triplicate 60-mm-diameter plastic dishes aiming for 80 to 100 colonies per dish. After 10 to 12 days of incubation, colonies were fixed with 10% formalin and stained with crystal violet. Colonies with  $>50$  cells were scored as a surviving colony.

**Cell Adhesion Assay.** Plastic plates (96 wells) were coated with 10  $\mu$ g/mL of collagen, laminin, fibronectin, and vitronectin (IWAKI, Chiba, Japan) in PBS (Invitrogen) for 2 hours at 37°C and then treated with 3% bovine serum albumin for 1 hour at 37°C, or were coated with only bovine serum albumin for negative control. The cells ( $2 \times 10^5$  cells/mL) in serum-free DMEM containing 0.1% bovine serum albumin were then added and incubated for 2 hours at 37°C. After removal of the medium, a 0.04% crystal violet solution was added and incubation was conducted for 10 minutes at room temperature. The wells were washed thrice with PBS and 20  $\mu$ L of Triton X-100 were added for permeabilization. Finally, distilled water was then added for a total quantity of 100  $\mu$ L, and the number of adherent cells was assessed with a microplate reader (measurement wavelength = 550 nm and reference wavelength = 630 nm).

**Flow Cytometry.** For  $\alpha$ V $\beta$ 3 and  $\beta$ 1 integrin analysis, cells in DMEM supplemented with 1% fetal bovine serum and 0.03% sodium azide were incubated with a monoclonal antibody against mouse monoclonal antibody  $\alpha$ V $\beta$ 3 and  $\beta$ 1 (Chemicon), for 30 minutes at 4°C. After washing with DMEM as described above, the cells were incubated with FITC-conjugated mouse IgG (DAKO, Copenhagen, Denmark) for 30 minutes at 4°C. After washing, cells were resuspended with the same medium and analyzed using a FACSCalibur (Beckton Dickson, Heidelberg, Germany) with CellQuest software (Beckton Dickson). Finally, cell surface fluorescence for individual integrin receptors was obtained.

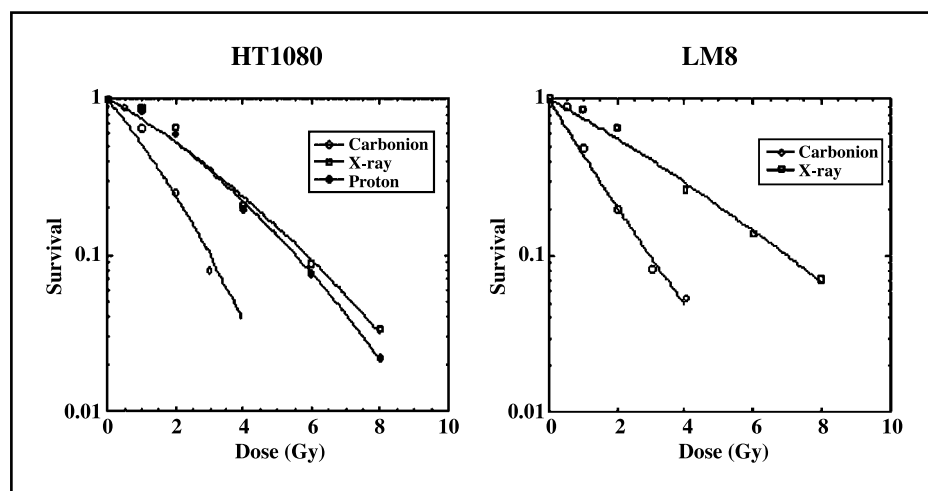
**Chemotaxis Assay.** Chemotaxis was assessed with a 48-microwell chemotaxis chamber (Neuro Probe, Gaithersburg, MD) that was set a

polycarbonate filter of 8- $\mu$ m pores coated with 10  $\mu$ g/mL fibronectin. The cells were trypsinized, resuspended in 0.1% bovine serum albumin and adjusted to a final concentration of  $1 \times 10^6$  cells/mL. The cells ( $5 \times 10^4$ ) were added to the upper well, which was placed into a lower well containing medium with 10% fetal bovine serum as a chemoattractant. After 3 hours of incubation at 37°C, cells remaining on the upper membrane surface were removed with a cotton swab. The cells that had migrated to the bottom of the filter were fixed with formalin and stained with hematoxylin. Cell migration was quantitated by counting the number of stained nuclei in four random fields at 20 $\times$  magnification with a microscope.

**Matrigel Invasion Assay.** Invasion of cancer cells was assessed by measuring the invasion of cells through transwell inserts with 8- $\mu$ m pores coated with Matrigel (Becton Dickinson). Irradiated cells were trypsinized, washed twice with DMEM supplement with 0.1% bovine serum albumin, and 200  $\mu$ L of cell suspension ( $5 \times 10^5$  cells/mL) per condition were added to the upper well. DMEM supplement with 10% fetal bovine serum (700  $\mu$ L) as a chemoattractant was added to the lower well. The number of cells that had invaded to the lower surface of the Matrigel-coated membrane was counted in four random fields under a microscope.

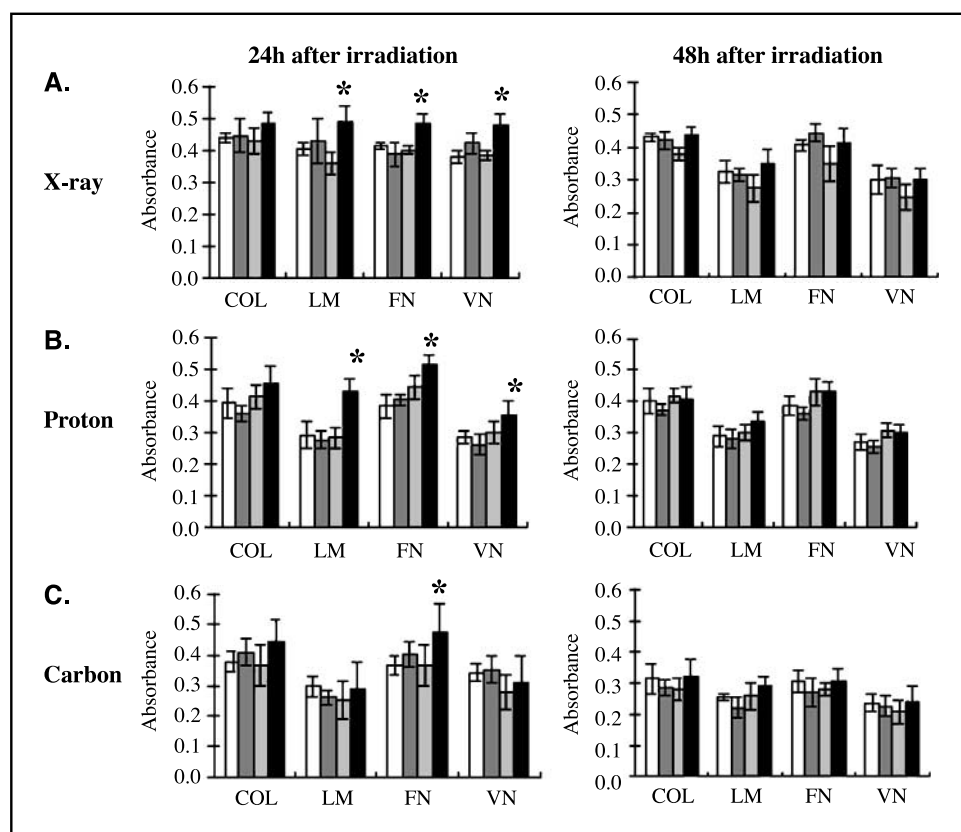
**Gelatin Zymography.** MMP-2 activity was analyzed as detailed elsewhere (22). After irradiation, cells were washed twice with PBS and incubated with serum-free DMEM for 24 hours. After the medium had been centrifuged to remove corpuscular material, supernatant was collected, frozen in liquid nitrogen, and stored at  $-80^\circ\text{C}$ . Samples were mixed with SDS sample buffer without heating or reduction and applied to 8% polyacrylamide gels containing 0.1% gelatin. After electrophoresis, gels were renatured by soaking for 45 minutes at room temperature in 2.5% Triton X-100 with gentle agitation and then incubated for 12 hours at 37°C in buffer containing 5 mmol/L  $\text{CaCl}_2$  and 1  $\mu$ mol/L  $\text{ZnCl}_2$ . Gelatinolytic activity made the bright bands visible at  $M_r$  72,000 for the pro form and  $M_r$  62,000 for the active form of MMP-2.

**Animal and Tumor Model.** LM8 cells were irradiated with 290 MeV/nucleon carbon ion beams or 4 MV X-ray (proton irradiation was not done because of restricted irradiation time). Cells were harvested by treatment with trypsin-EDTA (Invitrogen), washed twice with serum-free DMEM, and suspended in serum-free DMEM. Irradiated LM8 mouse osteosarcoma cells,  $10^5$  cells in 0.05 mL, were injected s.c. into the hind limbs or inoculated into tail vein of 8- to 10-week-old female specific pathogen-free C3H/HeJ mice (Charles River, Yokohama, Japan). In s.c. tumor, tumor volume ( $\text{mm}^3$ ) was measured with calipers and calculated according to the formula:  $1/2 \times \text{length} \times \text{width}^2$ . Mice injected s.c. or i.v. were euthanized 15 or 30 days after injection. Lung tumor formation was observed under a dissecting stereomicroscope, and the number of lung tumors was counted. These experiments were repeated twice.



**Figure 1.** Clonogenic survival curves after photon, proton, or carbon beam irradiation for cancer cells. Surviving fractions against physical doses were plotted and fitted to surviving curves using the following linear-quadratic model:  $\text{SF} = \exp(-\alpha D - \beta D^2)$ , where SF is the surviving fraction and  $D$  is the physical dose. Survival curve for proton irradiation was examined only in HT1080 cells.

**Figure 2.** Effects of irradiation on adhesion of cancer cells to collagen (COL), laminin (LM), fibronectin (FN), or vitronectin (VN). *A*, cells were untreated or irradiated with photon beams. *White bar*, untreated control cells; *dark gray bar*, cells irradiated at 0.5 Gy; *light gray bar*, cells irradiated at 2 Gy; and *black bar*, cells irradiated at 8 Gy. *B*, cells were untreated or irradiated with proton beam. *White bar*, untreated control cells; *dark gray bar*, cells irradiated with 0.5 Gy; *light gray bar*, cells irradiated with 2 Gy; and *black bar*, cells irradiated with 8 Gy. *C*, cells were untreated or irradiated with carbon ion beam. *White bar*, untreated control cells; *dark gray bar*, cells irradiated at 0.2 Gy; *light gray bar*, cells irradiated at 1 Gy; and *black bar*, cells irradiated at 4 Gy. *Columns*, mean; *bars*,  $\pm$  SD. \*,  $P < 0.05$  (Student's *t* test, compared with untreated cell).



**Statistics.** The results were expressed as mean values with SDs of at least three independent experiments, except indicated elsewhere. The statistical significance was tested by means of Student's *t* test or ANOVA where appropriate.  $P < 0.05$  was considered statistically significant.

## Results

**Survival Curves of Cancer Cells.** To require biologically equivalent doses for each radiation quality, we first examined clonogenic survival using the colony formation assay. For HT1080 cells, the relative biological effectiveness values, calculated by the D10 relative to X-ray, were to be 1.1 for proton irradiation and 1.9 for carbon irradiation (Fig. 1). The corresponding relative biological effectiveness measured by the D10 relative to X-rays for the LM8 cells was 2.3 for carbon irradiation (Fig. 1). Therefore, in subsequent assays, we applied the physical doses of carbon ion or proton to half or the same physical doses for X-ray.

**The Expression Levels of Integrin and Adhesion to Extracellular Matrix.** Cell adhesion assays were done to assess tumor cell adhesion capabilities to extracellular matrix proteins (collagen, fibronectin, laminin, and vitronectin). The adhesion capability to fibronectin, laminin, and vitronectin of cells irradiated with 8 Gy for X-ray or proton had significantly increased 24 hours after irradiation (Fig. 2A and B). Irradiation with 4 Gy for carbon at 24 hours after irradiation showed a significantly higher attachment to fibronectin compared to the nontreatment controls (Fig. 2C). On the other hand, no significant changes were observed 48 hours after X-ray, proton, and carbon ion irradiation (Fig. 2A-C). Mean fluorescence intensity (% control) of integrins that plays crucial roles in cell adhesion to extracellular matrices, cell migration, and invasion was analyzed by flow cytometry analyses. The expression

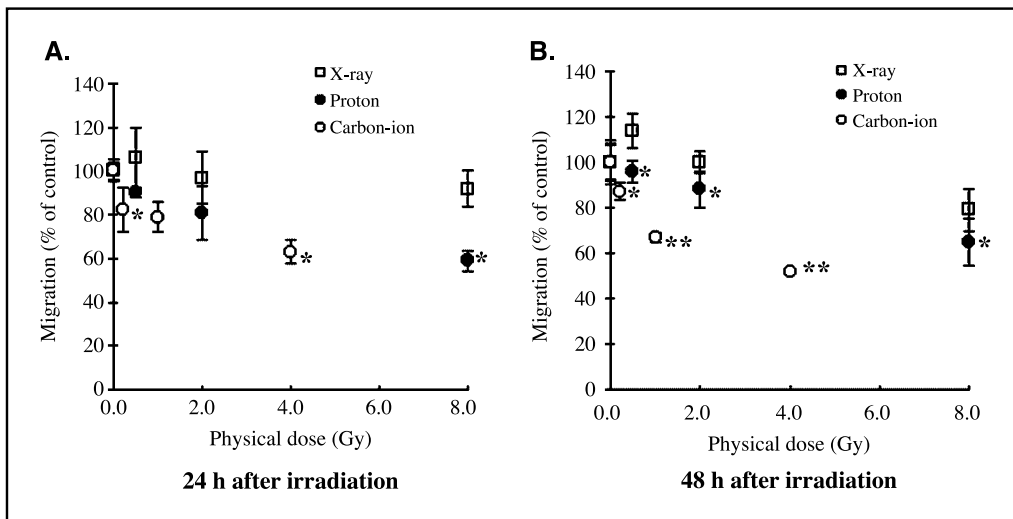
levels of  $\beta 1$  integrin did not show significant differences for X-ray, proton, and carbon ion irradiation at 24 hours after irradiation (Table 1). After delivery of more than 4 Gy of each type of irradiation, the amount of  $\beta 1$  integrin was increased, although not significantly. The expression levels of  $\alpha V\beta 3$  integrin were not changed by either proton or carbon ion irradiation (Table 1). However, for cells irradiated with 0.5 Gy of X-ray, the amount of  $\alpha V\beta 3$  integrin was significantly increased compared with untreated controls (Table 1).

**Effects of Irradiation on Cell Migration and Invasion.** Cell migration and invasion are fundamental components of tumor

**Table 1.** Effects of irradiation on the expression levels of  $\beta 1$  and  $\alpha V\beta 3$  integrin in cancer cells by flow cytometric analysis

Integrin		0.5 (0.2) Gy	2 (1) Gy	8 (4) Gy
$\beta 1$	X-ray	94 $\pm$ 7	107 $\pm$ 8	131 $\pm$ 21
	Proton	93 $\pm$ 2	94 $\pm$ 4	118 $\pm$ 11
	Carbon	106 $\pm$ 15	103 $\pm$ 16	115 $\pm$ 12
$\alpha V\beta 3$	X-ray	132 $\pm$ 10*	119 $\pm$ 20	102 $\pm$ 9
	Proton	95 $\pm$ 14	87 $\pm$ 8	98 $\pm$ 11
	Carbon	97 $\pm$ 9	101 $\pm$ 18	106 $\pm$ 6

NOTE: Number in parentheses represents the physical dose of carbon ion. \* $P < 0.05$  (Student's *t* test, compared with untreated cell).



**Figure 3.** Effects of irradiation on cell migration of HT1080 cells. HT1080 cells were exposed to X-ray (□), proton (●), or carbon (○). Vertical axis, no. migrated cells (%control); horizontal axis, physical dose. Points, mean; bars, ± SD. \*,  $P < 0.05$ ; \*\*,  $P < 0.01$  (ANOVA, compared particle beams with X-ray at similar killing doses).

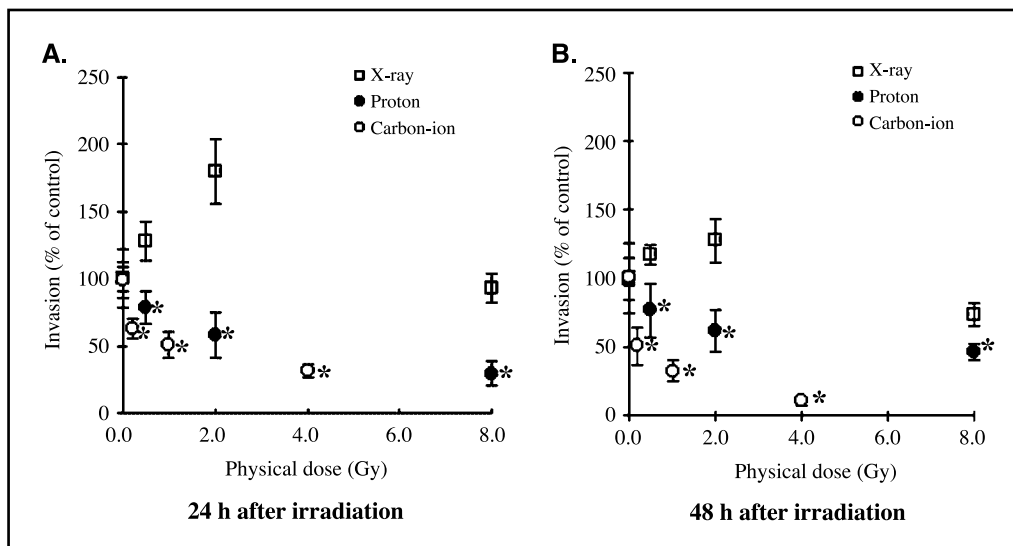
cell metastasis. To assess the effect of photon, proton, and carbon ion beams on cell motility, we examined the migration of malignant cells 24 and 48 hours after irradiation using chemotaxis assay. For proton as well as carbon ion irradiation, suppression of migration of irradiated cells became apparent at 24 and 48 hours after irradiation in a dose-dependent manner (Fig. 3A and B). On the other hand, an increase in migration was observed by lower dose (0.5 Gy) of X-ray irradiation at 48 hours after irradiation (Fig. 3B). At similar cell killing doses, proton or carbon particle irradiation, compared to X-rays, inhibited the migration (for carbon irradiation except 24 hours after 2 Gy,  $P < 0.05$ ; At 24 hours after 8 Gy or at 48 hours after proton irradiation,  $P < 0.05$ ).

We next focused on changes in the invasive capability of cancer cells after irradiation using the Matrigel invasion assay. Proton as well as carbon ion beam irradiation significantly reduced the invasion capabilities of irradiated cells (Fig. 4A and B). X-ray irradiation promoted cell invasion even at the dose levels below 2 Gy (Fig. 4A and B). Remarkably, invasive potentials of malignant cells were significantly increased by about 2-fold at 24 hours after X-ray irradiation at dose of 2 Gy (Fig. 4A). For cells irradiated with

8 Gy at 48 hours after X-ray irradiation, invasion capabilities were decreased as compared with untreated controls (Fig. 4B). Proton or carbon ion irradiation at comparable cell-killing doses resulted in significantly diminished invasion capabilities 24 or 48 hours after irradiation compared to that resulting from X-ray irradiation ( $P < 0.01$ ).

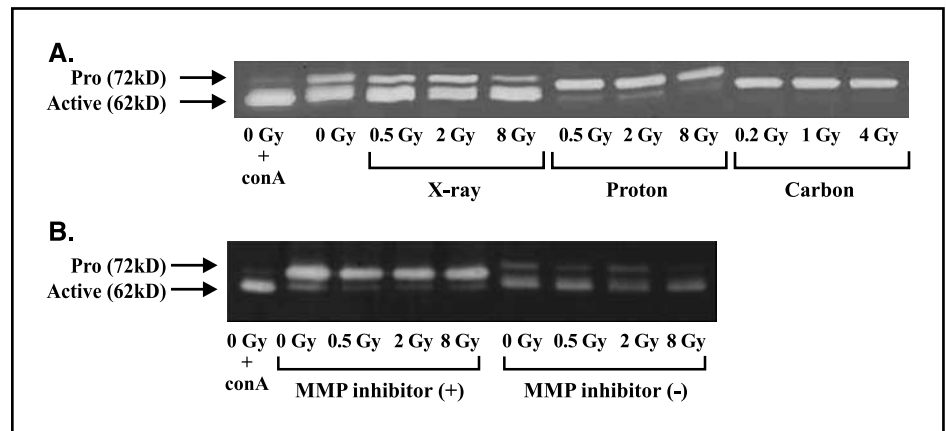
**Effects of Irradiation on MMP-2 Activity.** The process of tumor cell invasion and metastasis requires the degradation of connective tissue associated with vascular basement membranes and interstitial connective tissue. Therefore, MMP-2 activity required for tumor invasion was examined by gelatin zymography. Gelatin zymography revealed that proton and carbon ion irradiation strongly inhibited MMP-2 activity of cancer cells in a dose-dependent manner (Fig. 5A). For cells irradiated with X-ray, MMP-2 activity were not changed compared with untreated controls (Fig. 5A).

**The Influence of MMP Inhibitor on Photon-Enhanced Cell Invasion.** Particle irradiation inhibited invasion capability due to an association with inhibition of MMP-2 activity. Therefore, we investigated whether the MMP inhibitor could



**Figure 4.** Effects of irradiation on cell invasion of HT1080 cells. HT1080 cells were irradiated with X-ray (□), proton (●), or carbon (○). Vertical axis, no. invaded cells (% control); horizontal axis, physical dose. Points, mean; bars, ± SD. \*,  $P < 0.01$  (ANOVA, compared particle beams with X-ray at comparable cell killing doses).

**Figure 5.** Effects of irradiation on MMP-2 activity of cancer cells. Supernatants from untreated or irradiated cells were collected 24 hours after irradiation and analyzed by zymography for pro form (72 kDa) or active form of MMP-2 (62 kDa). Untreated samples in addition to concanavalin A (*conA*) were used for positive control. *A*, cells were untreated or irradiated with X-ray, proton, or carbon ion. *B*, cells were untreated or irradiated with X-ray in the presence or absence of the MMP inhibitor. MMP inhibitor (25  $\mu\text{mol/L}$ ) was added in condition medium just after irradiation.



prevent photon-induced increase in invasive potential. The addition of the MMP inhibitor GM6001 to the upper well resulted in a marked reduction of photon-induced invasion (Table 2). Furthermore, gelatin zymography showed that administration of GM6001 at concentration of 25  $\mu\text{mol/L}$  reduced the active form of MMP-2 (Fig. 5B).

**The Effect of Irradiated Cells on Metastatic Capabilities *In vivo*.** To investigate whether irradiated cells affect metastatic capabilities *in vivo*, LM8 osteosarcoma cells irradiated with X-ray or carbon ion were injected s.c. into right thighs or i.v. into tail vein of mice. In s.c. tumor, the tumor volumes obtained from irradiated cells were decreased as compared with those of untreated cells (Fig. 6A and B). There was no difference in the volume of tumors from cells treated with X-ray at doses between 2 and 10 Gy (Fig. 6A). However, for cells irradiated with carbon ion, tumor volume was decreased dose dependently (Fig. 6B). Pulmonary metastases of cancer cells irradiated with X-ray did not change in comparison with those of untreated controls (Fig. 6C), whereas treatment with carbon ion reduced the number of lung metastases in a dose-dependent manner (Fig. 6D).

For mice inoculated i.v., X-ray irradiation resulted in a 1.2-fold increase in the number of metastatic lung nodules in mice as compared to mice injected with untreated cells (Fig. 6E and F). However, a significant suppression of lung metastases was observed in cells irradiated with carbon ion (Fig. 6E and G).

**Table 2.** The influence of MMP inhibitor on photon-enhanced cell invasion

	0 Gy	0.5 Gy	2 Gy	8 Gy
Control media	100 $\pm$ 10	117 $\pm$ 7	107 $\pm$ 8	93 $\pm$ 10
+25 $\mu\text{mol/L}$ MMP inhibitor	80 $\pm$ 7	87 $\pm$ 11	76 $\pm$ 11	58 $\pm$ 5
Each treated versus control, <i>P</i>	<0.05	<0.05	<0.05	<0.05

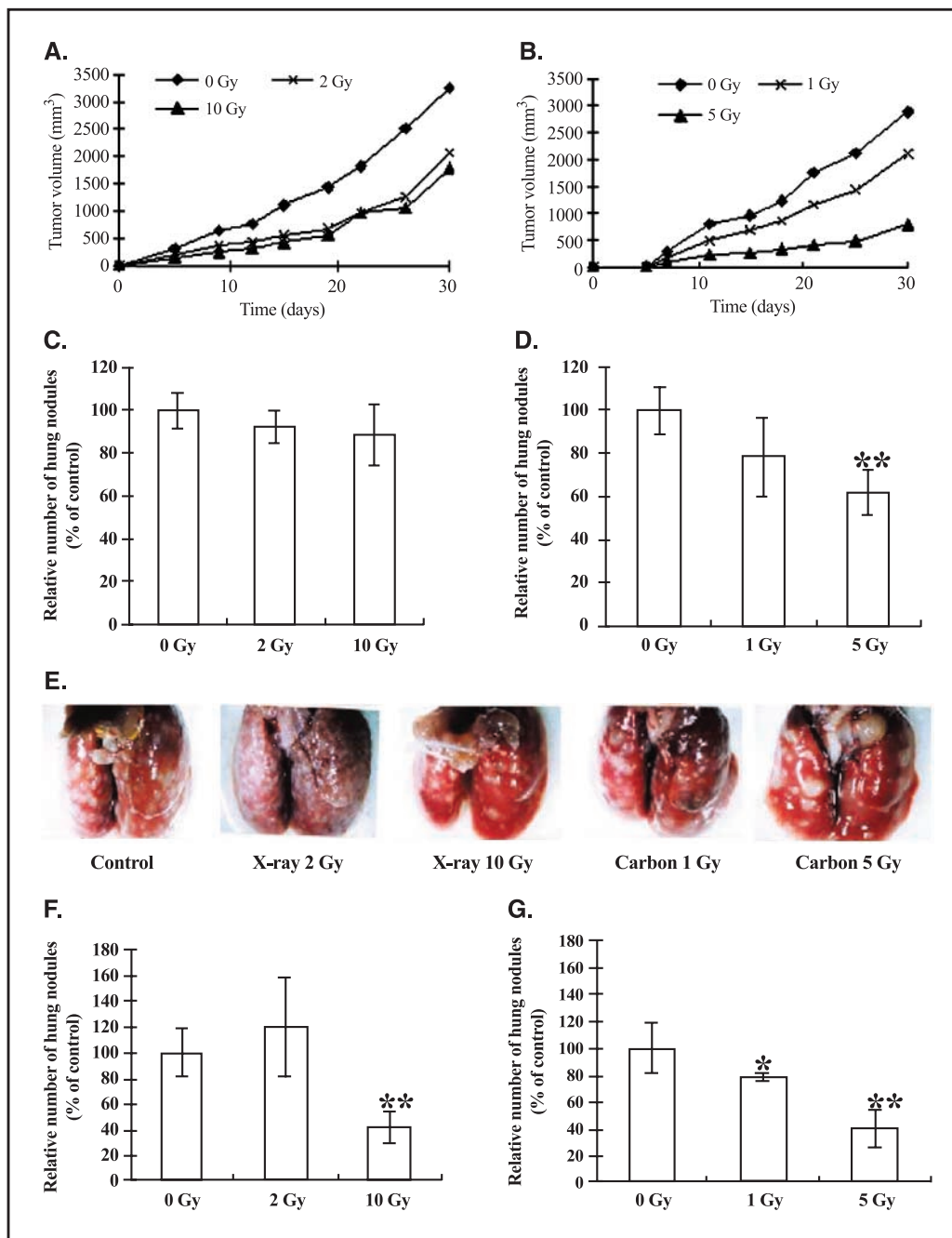
NOTE: Data were calculated with reference to untreated controls defined as a percentage scale.

## Discussion

Metastasis brings about the greatest threat to the survival and quality of life for cancer patients. The ultimate goal of cancer therapy is to treat the primary tumor and any underlying metastases. Particle radiotherapy such as proton and carbon ion has established its efficacy by demonstrating superb results (23–28). The advantage of particle beams over photon is superior distribution of radiation dose due to the physical characteristics, which makes it possible to spare normal tissues close to the target. However, the effects of particle beams on metastatic potential of cancer cells are not yet well understood. We hypothesized that particle beams might inhibit metastatic potential for ion beam-specific biological effects and first focused on the *in vitro* models including adhesion, migration, invasion, and the expression level or activity of molecules related to metastasis such as  $\alpha\text{V}\beta3$ ,  $\beta1$  integrin, and MMP-2.

Various factors are related to metastatic potentials. Changes in integrin expression level are likely to affect cell adhesion closely linked cell functions. X-ray, proton, and carbon ion irradiation of more than 4 Gy was seen to increase significantly cell adhesion capability to extracellular matrix significantly. Cordes et al. (29, 30) showed that radiation-induced increase in adhesion capacity could be modulated by radiation-induced increase in  $\beta1$  integrin expression. However, our findings showed that the expression levels of  $\beta1$  integrin were increased ( $\geq 4$  Gy irradiation) but did not show significant differences among X-ray, proton, and carbon ion irradiation. The reason for these discrepant results may be that the use of flow cytometry does not enable us to detect  $\beta1$  integrin affinity but only the expression level of  $\beta1$  integrin. Integrin affinity for extracellular matrix can be regulated by intracellular signals such as the Ras-, R-Ras- and Rap1-GTPases (31, 32).  $\beta1$  Integrin transduces biochemical signals from the extracellular environment, especially with respect to cell survival. It seems likely that radiation ( $\geq 4$  Gy) may activate  $\beta1$  integrin affinity and thus leading radiation-induced ( $\geq 4$  Gy) increase in adhesion capacity due to cell survival.

Cell migration and invasion are fundamental components of tumor cell metastasis. Wild-Bode et al. (9) reported that sublethal dose of X-ray irradiation induced the expression levels of the  $\alpha\text{V}\beta3$  integrin of glioblastoma and led to enhancement of cell migration. We confirmed that X-ray irradiation promotes cell migration capabilities concomitant with the up-regulation of  $\alpha\text{V}\beta3$  integrin at lower dose level. However, our study showed that both proton and carbon ion irradiation significantly decreased cell migration



**Figure 6.** Metastatic capabilities of cancer cells irradiated with photon or carbon beam *in vivo*. Growth curves in subcutaneous tumors of LM8 osteosarcoma cells unirradiated, irradiated with X-ray (A), or carbon ion (B). The number of lung metastases treated with X-ray (C), or carbon ion (D) in subcutaneous tumor. Representative of lung metastasis for mice injected intravenously (E). Therapeutic effects of cells irradiated with X-ray (F) or carbon ion (G) on experimental pulmonary metastasis from mice inoculated intravenously. Columns, mean; bars,  $\pm$  SD. \*,  $P < 0.05$  and \*\*,  $P < 0.01$  (Student's *t* test versus the untreated group).

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and invasion capabilities in a dose-dependent manner. Many studies have shown that MMP-2 plays a critical role in tumor invasion. There have been many reports on the enhancement of MMP-2 activity by X-ray irradiation (9, 12, 33, 34). One of the mechanisms of this enhancement is that the activation of wild-type p53 by photon irradiation and the resulting increase in MMP-2, which can promote radiation-induced metastasis. Bian and Sun (35) reported that the 5' flanking region of the *MMP-2* gene contains a perfect p53 binding sequence and that the binding of wild-type p53, but not mutant p53, to this site up-regulates *MMP-2* gene expressions. In a previous study, for HT1080 cells expressed wild-type p53,  $\gamma$ -ray irradiation with doses from 4 to 15 Gy up-regulated this expression (36). Our study showed that MMP-2 was

strongly inhibited by carbon ion and proton irradiation. Therefore, invasion capabilities of irradiated cells were significantly suppressed by particle beams. Furthermore, we confirmed that MMP inhibitor blocked the photon-enhanced invasion of cancer cells. Our results concur with Wild-Bode's report that administration of *o*-phenantroline that is one of the MMP inhibitors significantly inhibited photon-induced invasiveness. Asakawa et al. (37) showed that p53-dependent radiation-induced growth inhibition of SAS tongue carcinoma cells transplanted into nude mice was observed following X-ray irradiation but not carbon ion irradiation. Our finding suggests that particle beam irradiation is not affected by p53 status.

The phenomena underlying the suppression of metastatic capability by particle irradiation *in vitro* were studied further by

investigating metastatic potentials of cancer cells irradiated with carbon ion or photon beams *in vivo*. For mice inoculated s.c. or i.v., treatment with carbon ion reduced the number of lung metastases in a dose-dependent manner as compared with untreated controls. For several experimental tumors, inadequate X-ray radiation resulted in an increase in metastasis (38). One possible explanation for this increase is that radiation-induced DNA changes increase the metastatic potential of cancer cells (39). Our data suggest that carbon ion irradiation induced DNA changes which suppressed the metastatic capabilities of tumor cells, leading to suppression of pulmonary metastases *in vivo*. This may have been caused by carbon ion irradiation producing a higher proportion of double-strand DNA breaks than does X-ray irradiation.

In this study, the focus was to elucidate the effects of particle beam on metastatic potential of cancer cells. However, little is known about the basic radiobiological effects of particle beam except for the end point of cell survival, especially about the effects on metastatic capabilities. To date, a few groups have reported on the effects of particle beams on cell functions associated with metastatic capabilities. Our group showed that carbon ion irradiation inhibited MMP-2 activity and down-regulated  $\alpha V\beta 3$  integrin, thus leading to inhibition of *in vitro* angiogenesis (17). Ando et al. (40) reported that the induction by carbon ion irradiation of vascular endothelial growth factor that plays an important role in tumor growth and metastasis. However, lung carcinoma cells irradiated with carbon ion induced vascular endothelial growth factor mRNA expression and increased protein levels dose dependently. Particle therapy still has much room to be studied for optimum use in clinical oncology compared with conventional photon beam treatment. Further intensive studies are also necessary to elucidate the relevant molecular mechanisms specifically related to particle irradiation. In future experiments, other carcinoma cell lines will be examined to confirm that this phenomenon is not specific to one cell line.

The phenomena we observed in this study have two significant impacts on the clinic. First, with advent of recent high precise modality such as intensive modulated radiation therapy, radiation oncologists have been focusing on making the radiation field as small as possible to the clinical target volume. There may be a risk that excellent local controls can be hampered by later increase of distant metastasis. Then, we need individualized radiation field based on such biological behavior of each cancer cell. Second, particles such as proton and carbon may have totally different mechanism of action on cell migration and invasion, because these functions were significantly inhibited even at lower doses of particle. These significant differences in cell functions may be

caused by differences in biological mechanisms between particle and electromagnetic wave but cell-killing effect concerning cell survival evaluated with colony formation assay of proton are similar to that of photon.

Photon radiation therapy should be asked with some caution. Lower photon irradiation promotes cell migration and invasive capabilities. However, metastatic capabilities of cancer cells irradiated with 8 Gy of photon beams did not change in comparison with those of untreated controls. The clinical implications reported by Wild-Bode et al. (9) are that alterations in the fractionation of radiotherapy for human glioblastoma multiform may need to be considered and that inhibitors of migration and invasion may prevent irradiation-induced dissemination of glioma cells from the target volume of irradiation when given during radiotherapy. In addition to these implications, we suggest that not only dose escalation that can eradicate tumors is needed but also examination of the individual radiation field margins, by considering cell migration and identifying microscopic diseases by means of molecular imaging is needed.

To summarize, our study found that particle irradiation decreased cell migration and invasion in a dose-dependent manner and strongly inhibited MMP-2 activity *in vitro*. *In vivo*, treatment with carbon ion reduced the number of lung metastases in a dose-dependent manner. On the other hand, lower X-ray irradiation facilitated cell migration and invasion concomitant with up-regulation of  $\alpha V\beta 3$  integrin *in vitro*.

In conclusion, these data suggest that particle irradiation suppresses metastatic potential even at lower dose whereas photon irradiation promotes cell migration and invasive capabilities at lower dose level. These findings provide preclinical rationales that particle radiotherapy may be superior to conventional photon beam therapy in possible preventive effects on metastases of irradiated malignant tumor cells.

## Acknowledgments

Received 3/23/2004; revised 10/7/2004; accepted 10/31/2004.

**Grant support:** Japan Society for the Promotion of Science Grants-in-Aid for Scientific Research 13670936 and 16390338, Ministry of Health, Labor and Welfare in Japan Grant-in-Aid for Cancer Research 14-6, and a research project with heavy ions at National Institute of Radiological Science-Heavy Ion Accelerator in Chiba (12B621 and 13B132).

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We thank Sakae Yamamoto, Ayako Madachi, Ryuji Ikeda, Masaki Tanabe, Eri Adachi, Kouki Okita, Noriko Ohnishi, and Kimiko Sameshima at the Molecular Pathology Laboratory and Ayaka Kihara at the Radiation Oncology Laboratory for excellent technical support; Dr. Mitsuyuki Abe at Hyogo Ion Beam Medical Center for critical reading of the article and helpful discussions; and the cooperation of all staffers at Hyogo Ion Beam Medical Center in performing proton irradiation.

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