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Heavy Ion Irradiation Inhibits *in Vitro* Angiogenesis Even at Sublethal Dose[1]

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ABSTRACT

Angiogenesis is essential for tumor growth and metastasis. Because endothelial cells are genetically stable, they rarely acquire resistance to anticancer modalities, and could, thus, be a suitable target for radiation therapy. Heavy ion radiation therapy has attracted attention as an effective modality for cancer therapy because of its highly lethal effects, but the effects of heavy ion irradiation on *in vitro* cell function associated with angiogenesis have not been reported. Our study found that *in vitro* angiogenesis was inhibited by high linear energy transfer carbon ion irradiation even at sublethal dose (0.1 Gy). ECV304 and HUVEC human umbilical vascular endothelial cells were irradiated with 290 MeV carbon ion beams of approximately 110 keV/μm or 4 MV X-ray of approximately 1 keV/μm. Their adhesiveness and migration to vitronectin or osteopontin were inhibited, and capillary-like tube structures in three-dimensional culture were destroyed after carbon ion irradiation concomitant with the inhibition of matrix metalloproteinase-2 activity, down-regulation of $\alpha V\beta 3$ integrin, which is one of the adhesion molecules, slight up-regulation of membrane type1- matrix metalloproteinase, and significant up-regulation of tissue inhibitor of metalloproteinase-2. On the other hand, sublethal X-ray irradiation promoted migration of endothelial cells, and the capillary-like tube structure in three-dimensional culture progressed even after 16 Gy irradiation. These results provide an implication that heavy ion beam therapy could be superior to conventional photon beam therapy in preventive effects on *in vitro* angiogenesis even at sublethal dose, and might inhibit angiogenesis *in vivo*.

INTRODUCTION

Angiogenesis, the formation of new capillaries from pre-existing vessels, is essential for tumor progression and metastasis (1–6). This event is a complex phenomenon involving the proliferation and migration of capillary endothelial cells, remodeling of vascular extracellular matrix, and tubule formation (7). MMPs[3] play important roles on *in vitro* and *in vivo* angiogenesis, but their roles are very complicated. MMP-2 plays a critical role in angiogenesis (8–10). One of the mechanisms of this is that MMP-2 directly binds to $\alpha V\beta 3$ integrin and, thus, localizes in a proteolytically active form to the cell surface (11). However, O'Reilly *et al.* (12) demonstrated that MMP-2 was responsible for the production of angiostatin and, therefore, possibly suppresses angiogenesis. MT1-MMP is also essential for MMP-2 activity (13–15) and possesses gelatinolytic activity itself (16). On the other hand, TIMP-2, which is a constitutive inhibitor, inhibits MMP-2 activity (17). Thus, some MMPs can potentiate *in vitro* and *in vivo* angiogenesis, and other MMPs can be negative regulators.

Radiation therapy is important for the treatment of many human cancers but is occasionally unsuccessful because of tumor radiation resistance (18). Kinzler and Vogelstein (19) reported that cancer cells acquire resistance to hormonal therapy and chemotherapy because they are genetically unstable. On the other hand, endothelial cells are genetically stable (20, 21), making them a potentially suitable target for radiation therapy. To investigate this, we focused on *in vitro* angiogenesis models.

Previous studies have shown that X-ray irradiation can inhibit the proliferation of vascular endothelial cells (22, 23). However, Sonveaux *et al.* (24) demonstrated that low-dose irradiation of X-ray to endothelial cells could induce nitric oxide-mediated pathways leading to migration of endothelial cells and organization in vascular network. In addition, MMP-2 was activated in lung epithelial cells (25), indicating that angiogenic potential may persist after X-ray irradiation.

Heavy ion radiation therapy has attracted attention as an effective modality for cancer therapy because of its beneficial physical characteristics and its highly lethal effects even on radioresistant tumors (26). However, the effects of heavy ion beams on *in vitro* cell function associated with angiogenesis have not been reported.

Here we report that heavy ion irradiation inhibits *in vitro* angiogenesis even at sublethal dose.

MATERIALS AND METHODS

Reagents and Cell Culture. ECV304 and HUVEC human endothelial cells were used to analyze the effect of irradiation on *in vitro* angiogenesis systems. ECV304 cell line was obtained from American Type Culture Collection (Rockville, MD). HUVEC cells were purchased from Kurabo (Kurashiki, Japan) and cultured in MCDB131 (Invitrogen Corp., Carlsbad, CA) supplemented with 10% FBS (Life Technologies, Inc., Gaithersburg, MD), 2 mM L-glutamine, 0.001% recombinant human fibroblast growth factor, 0.1% hydrocortisone, 0.03% amphotericin B (Dainihonseiyaku, Osaka, Japan) and 1% penicillin/streptomycin (Life Technologies, Inc.) and maintained at 37°C in an atmosphere of 5% CO_2. ECV304 cells were cultured in DMEM (Nihonseiyaku, Tokyo, Japan) supplemented with 10% FBS and 1% penicillin/streptomycin, and maintained under the same condition as the HUVEC cells. For acquisition of the conditioned medium of HUVEC and ECV304 cells, the cells were grown in their respective medium to subconfluent monolayers, washed with phosphate-buffered NaCl solution (PBS), incubated with serum-free DMEM for 24 h, irradiated, left for 48 h after irradiation, and then incubated. Supernatant was harvested and stored at −80°C.

Irradiation. ECV304 and HUVEC cells were grown to 80% confluence in their respective medium and irradiated with 290 MeV Heavy Ion Medical Accelerator in Chiba carbon ion beams of approximately 110 keV/μm or 4 MV X-ray of approximately 1 keV/μm. Cells treated by carbon ion beams or untreated cells were transported by bullet train in a room temperature without injecting CO_2 from the National Institute of Radiological Sciences in Chiba to Osaka University in Suita. It took about 4 h. Cells were then incubated at 37°C in an atmosphere of 5% CO_2 for 24 or 48 h (including transportation time) before each assay. On the other hand, cells treated or untreated with X-ray in Osaka University were directly incubated without transportation in the same condition as above for 24 or 48 h before each assay.

Flow Cytometry. For $\alpha V\beta 3$ integrin analysis, ECV304 cells were treated as already described, washed with PBS, incubated with trypsin-EDTA (Life Technologies, Inc.) for 1 min at 37°C, harvested, and dissolved in DMEM supplemented with 1% FBS and 0.03% NaN_3. A total of 2×10^5 cells were

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[3] The abbreviations used are: MMP, matrix metalloproteinase; MT1, membrane type 1; TIMP, tissue inhibitor of metalloproteinase; HUVEC, human umbilical vascular endothelial cell; FBS, fetal bovine serum.

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incubated with 1 μg of $\alpha V\beta 3$ mouse monoclonal antibody (Chemicon, Temecula, CA) at 4°C for 30 min, washed twice with DMEM supplemented with 1% FBS and 0.03% NaN_3, incubated with 1 μg of goat IgG monoclonal antibody under the same conditions as before, and analyzed with a FACScaliber flow cytometer using Cell Quest acquisition and analysis software.

Cell Adhesion Assay. A 96-well plate was coated with vitronectin and osteopontin (1 μg/ml; Iwaki, Chiba, Japan), incubated for 2 h at 37°C in an atmosphere of 5% CO_2, and blocked with 3% BSA (Sigma Chemical, St. Louis, MO) for 2 h. The irradiated cells (2×10^5 cells/ml) were harvested with trypsin-EDTA, dissolved in 0.1% BSA, plated in the wells, incubated for 2 h under the same conditions as before, then stained with Crystal violet (0.04%) and washed twice with PBS. After the addition of DMSO, the cells were left at room temperature for 10 min. Distilled water was then added, and the number of adherent cells was assessed with a microplate reader (measurement wavelength 550 nm and reference wavelength 630 nm).

Boyden Chamber Assay. Cells were collected by using trypsin-EDTA in PBS and suspended with serum-free medium containing 0.1% BSA after the cells had been washed with the same medium. Migration of HUVEC and ECV304 cells was assessed by using 8-μm pores. A filter was placed on a 24-well plate and coated with vitronectin for 30 min at room temperature. DMEM supplemented with 10% FBS was used as a chemoattractant into the lower wells. Three $\times 10^4$ of untreated or treated cells were plated onto the filter membrane and then allowed to migrate through the membrane at 37°C in an atmosphere of 5% CO_2. After 3 h, cells that had not migrated were scraped off with a cotton swab, and the membrane filter was removed with a blade. Cells that had migrated to the bottom side of the membrane were fixed with 10% formalin and then stained with H&E solution. The number of cells that had migrated to the lower side through the pores was counted with a microscope.

Gelatin Zymography. MMP-2 activity was analyzed by zymography. Supernatants of untreated or treated cells were harvested and separated by 12% SDS-PAGE containing 0.1% gelatin without denaturing agents. Gels were washed for 60 min in 10% Triton X-100 and then incubated for 24 h at 37°C in 50 mM Tris-HCl (pH7.5), 5 mM $CaCl_2$, and 1 μM $ZnCl_2$ to allow the gelatinases to digest the gelatin structure. Gels were stained with 0.1% Coomassie Brilliant Blue R-250, and then destained with 10% acetic acid and 10% methanol. Gelatinolytic activity made the bright bands visible at M_r 72,000 for the pro form, M_r 65,000 for the intermediate form, and M_r 62,000 for the active form of MMP-2.

Western Blot. For the preparation of whole-cell lysates, the cells were rinsed in PBS; harvested; centrifuged; lysed in 10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 1 mM phenylmethylsulfonyl chloride/isopropanol, and 1 μg/ml pepstatin/methanol for 60 min on ice; and again centrifuged for 10 min. The lysed proteins were then separated by 10% SDS-PAGE and electroblotted on polyvinylidene difluoride membranes (Bio-Rad, Hercules, CA). After blocking for 1 h in PBS supplement with 5% skim milk, immunodetection of MT1-MMP (M_r 65,000) and TIMP-2 (M_r 21,000) were performed with antimouse monoclonal antibody (1:500; Calbiochem, San Diego, CA) or antirabbit polyclonal antibody (1:1000; Chemicon). Antirabbit or antimouse IgG (1:3000) and enhanced chemiluminescence (Amersham Pharmacia Biotech, Aylesbury, United Kingdom) were used for detection.

Collagen-embedded Culture Method. Collagen gel culture kit (Nitta gelatin Inc., Osaka, Japan) was used to analyze the capillary-like tube formation. For preparation of collagen gel solution, type I-A collagen, 10 × Ham's F12 medium and reconstitution buffer (containing 20 mM HEPES in NaOH liquid) were mixed on ice in the ratio of 8:8:1, respectively. We dispensed 0.5 ml of this solution into a 24-well plate as a base layer and allowed it to polymerized at 37°C. The irradiated cells were harvested, added into the solution to achieve a final concentration of 2×10^3 cells/well, 0.5 ml of it plated onto the base layer of each well, and then incubated for 20 min at 37°C to congeal the gels. After congealing the gels, 0.5 ml of DMEM supplemented with 10% FBS and 1% penicillin/streptomycin was added on the cell layer and allowed to incubate for 10 days at 37°C in an atmosphere of 5% CO_2. The new capillary tube formations were observed using an inverted phase-contrast microscopy, and their images in which some cells or tubes existed were randomly captured by a video camera system. The quantitation was performed by analyzing cumulative pixel sizes of capillary tubes in the images that correspond to their length and width of capillary tubes ($n = 4$).

Statistics. The data were calculated as mean values and SDs. The statistical significance was tested by Student's t test.

RESULTS

The Expression Level of $\alpha V\beta 3$ Integrin, and Adhesion to Vitronectin and Osteopontin. The expression level of $\alpha V\beta 3$ integrin of ECV304 cells was reduced by carbon ion irradiation in a dose-dependent manner 24 and 48 h after irradiation (Fig. 1*A*). The adhesiveness to vitronectin and osteopontin of ECV304 cells was decreased significantly (Fig. 2*A*). On the other hand, X-ray irradiation temporally increased $\alpha V\beta 3$ integrin of ECV304 cells in a dose-dependent manner 24 h after irradiation (Fig. 1*B*). There was no

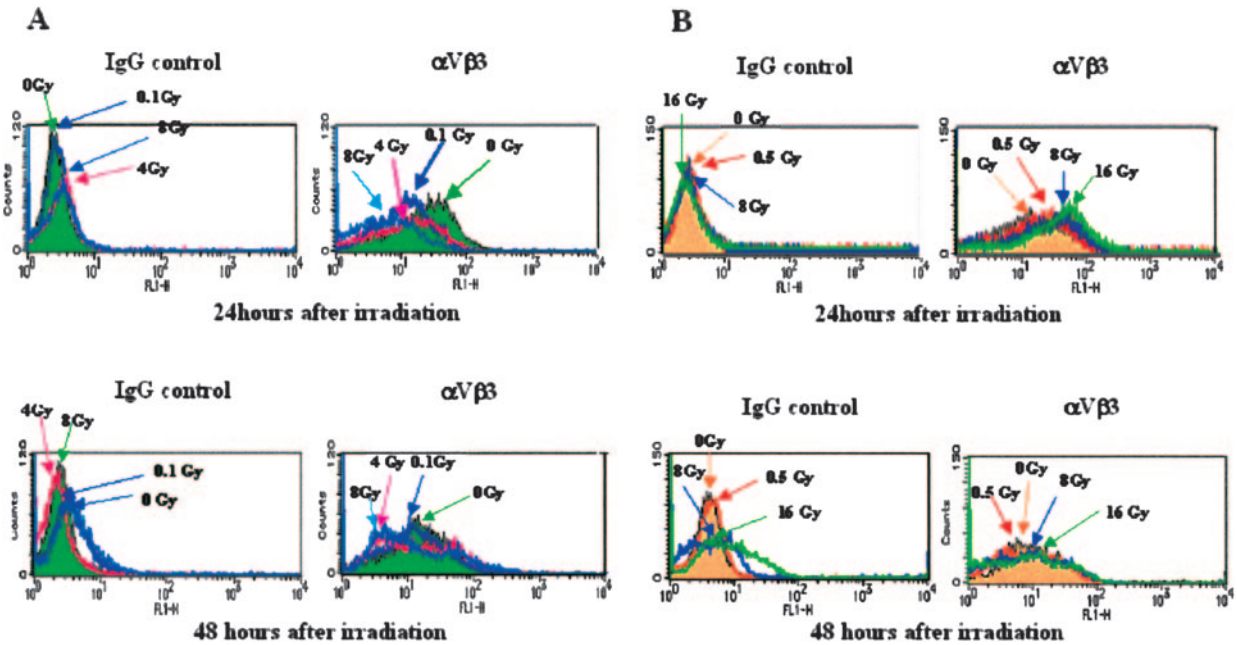

Fig. 1. Effects of irradiation on the expression levels of $\alpha V\beta 3$ integrin in endothelial cells by flow cytometric analysis. The number of cells is shown on the *vertical axis*, the logs of the integrin expression level on the *horizontal axis*. A cell group on the *right* correlates with a high level of expression. *A,* ECV304 cells were untreated or treated with carbon ion beams. *B,* ECV304 cells were untreated or treated with X-ray beams.

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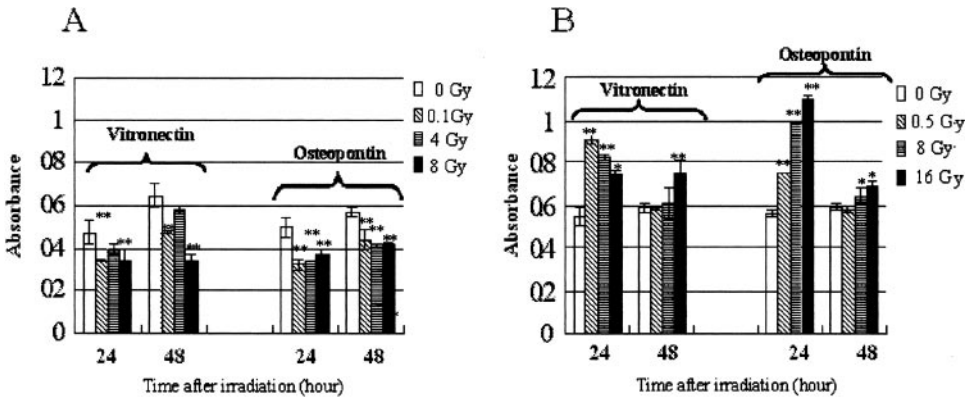

Fig. 2. Effects of irradiation on adhesion of ECV304 cells to vitronectin or osteopontin. *A,* ECV304 cells were untreated or treated with carbon ion beams. □, untreated control cells; ▨, cells treated at 0.1 Gy; ▤, cells treated at 4 Gy; ■, cells treated at 8 Gy. *B,* ECV304 cells were untreated or treated with X-ray beams. □, untreated control cells; ▨, cells treated at 0.5 Gy; ▤, cells treated at 8 Gy; ■, cells treated at 16 Gy. Data are expressed as mean cell counts ($n = 3$); *bars,* ±SE. *, $P < 0.05$; **, $P < 0.01$.

change in $\alpha V\beta 3$ expression 48 h after irradiation of 16 Gy, and a slight decrease was observed after 0.5 Gy and 8 Gy of irradiation. The adhesiveness to vitronectin and osteopontin of ECV304 cells was increased significantly 24 h after X-ray irradiation (Fig. 2*B*), but there was no change in adhesiveness at 0.5 Gy and 8 Gy 48 h after irradiation. Only 16 Gy irradiation increased adhesiveness.

Effects of Irradiation on Cell Migration. Carbon ion irradiation inhibited migration of HUVEC and ECV304 cells to vitronectin by >90% at both sublethal doses and doses that are known to significantly reduce clonogenic survival (Fig. 3*A*). This tendency continued up to 48 h after irradiation. The migration was not altered 24 h after X-ray irradiation (Fig. 3*B*), but irradiation of <8 Gy promoted migration to vitronectin, whereas 16 Gy irradiation inhibited it by 25%.

Effects of Irradiation on MMP Activity and Expression. Because MMP activity is thought to be required for migration of endothelial cells, we next examined whether irradiation altered MMP-2 activity. Gelatin zymography showed that carbon ion irradiation inhibited MMP-2 activity of both ECV304 and HUVEC cells in a dose-dependent manner (Fig. 4*A*). In fact, the intermediate (M_r 65,000) and active form (M_r 62,000) of MMP-2 completely disappeared as a result of 8 Gy irradiation to ECV304 cells. In contrast, X-ray irradiation promoted MMP-2 activity in a dose-dependent manner (Fig. 4*B*).

MMP-2 is activated by MT1-MMP on the cell surface, resulting in promotion of angiogenesis, whereas TIMP-2 inhibits MMP-2 activity. We next examined the expression levels of MT1-MMP and TIMP-2 of ECV304 cells. That of MT1-MMP was slightly increased by carbon ion irradiation (Fig. 5*A*), and TIMP-2 was increased significantly in a dose-dependent manner. X-ray irradiation remarkably increased MT1-MMP, but TIMP-2 was reduced at 0.5 Gy and 8 Gy (Fig. 5*B*).

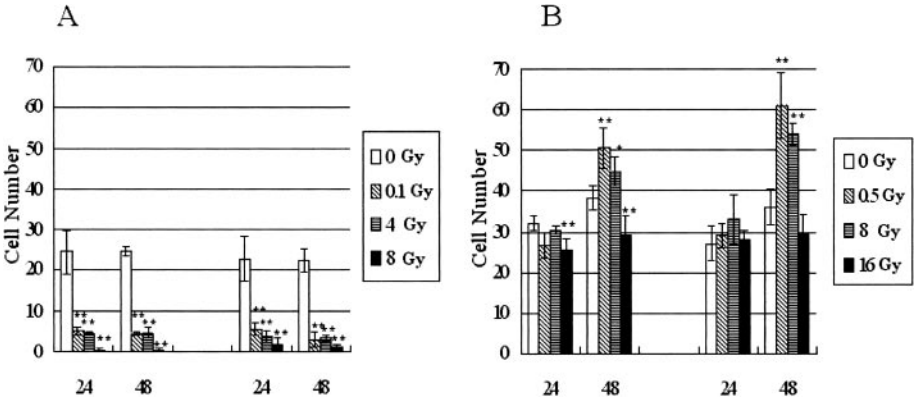

Fig. 3. Effects of irradiation on migration of ECV304 and HUVEC cells. The number of migrated cells is shown on the *vertical axis*. *A,* cells were untreated or treated with carbon ion beams. □, untreated control cells; ▨, cells treated at 0.1 Gy; ▤, cells treated at 4 Gy; ■, cells treated at 8 Gy. *B,* cells were untreated or treated with X-ray beams. □, untreated control cells; ▨, cells treated at 0.5 Gy; ▤, cells treated at 8 Gy; ■, cells treated at 16 Gy. Data are expressed as mean cell counts ($n = 3$); *bars,* ±SE. *, $P < 0.05$; **, $P < 0.01$.

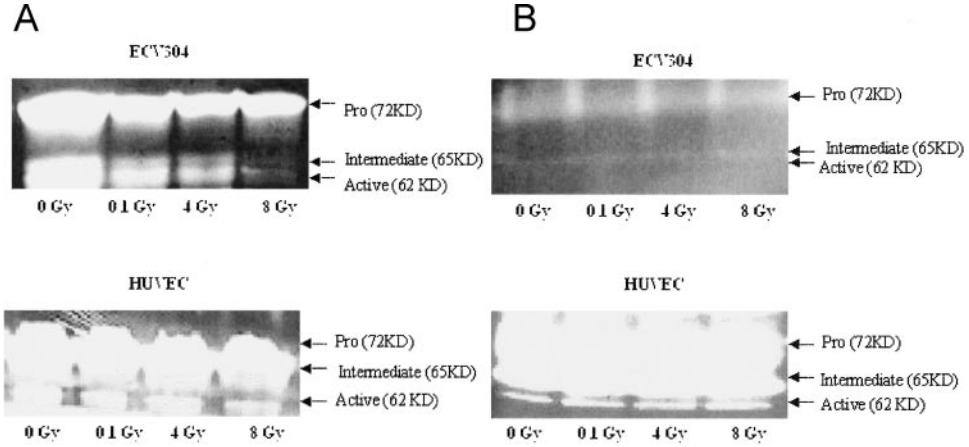

Fig. 4. Effects of irradiation on MMP-2 activity. Supernatants from untreated or treated cells were collected 48 h after irradiation, and analyzed by zymography for the proform (M_r 72,000), intermediate form (M_r 65,000), or active form of MMP-2 (M_r 62,000). *A,* cells were untreated or treated with carbon ion beams. *B,* cells were untreated or treated with X-ray beams.

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Effects of Irradiation on the Formation of Capillary-like Tube Structure. The capillary-like tube structure in three-dimensional culture of ECV304 cells was inhibited significantly by carbon ion irradiation in a dose-dependent manner (Fig. 6*A*). About 90% of the cells irradiated even at 0.1 Gy could be destroyed compared with unirradiated cells (Fig. 6*C*). On the other hand, X-ray irradiation of 0.5 Gy could not inhibit the tubes, and progression of cells was observed even after >8 Gy irradiation (Fig. 6*B*).

DISCUSSION

We investigated the effects of heavy ion irradiation on *in vitro* angiogenesis systems compared with these of X-ray irradiation. Because we transported cells that were irradiated by carbon ion beams as described in "Materials and Methods," we examined the effects of the transportation on cell damages in advance and confirmed that no significant damages were observed at least for cell survival (data not shown).

Many studies have shown that high linear energy transfer radiations are more effective than low linear energy transfer radiations, such as X-rays or γ-rays, for cell-killing effect (27–29). In fact, results with heavy ion beam therapy seem very promising for prostate carcinoma, head and neck cancer, and even for radioresistant tumors, although only a few clinical trials have been attempted to compare conventional photon radiotherapy with heavy ion radiotherapy (30). We hypothesized that heavy ion beams might inhibit angiogenesis, and first focused on the *in vitro* models including migration, the expression level or activity of relating molecules to angiogenesis such as αVβ3 integrin and MMPs, and three-dimensional capillary tube formation of endothelial cells.

Various factors are related to angiogenesis. αVβ3 integrin, which is a vitronectin and osteopontin receptor, is one of the candidates playing a critical role in angiogenesis. Brooks *et al.* (31) reported that a monoclonal antibody to αVβ3 blocked angiogenesis. Furthermore, MMP-2 binds directly with αVβ3 and, thus, localizes in a proteolytically active form to the cell surface, resulting in the promotion of angiogenesis (11). Wild-Bode *et al.* (32) reported that the expression levels of the αVβ3 integrin of glioblastoma cells were increased by X-ray irradiation, and led to enhancement of cell migration. This enhancement was abolished by αVβ3 monoclonal antibody, indicating that reduction αVβ3 integrin could inhibit cell migration. In our study, migration of endothelial cells was inhibited by carbon ion irradiation concomitant with the reduction of the expression levels of αVβ3. Furthermore, MMP-2 activity was also reduced by carbon ion beams. These phenomena seemed similar to the reports of Brooks *et al*. (11) and Wild-Bode *et al*. (32) in the blockade of αVβ3-suppressed cell migration and MMP-2 activity.

MMP-2 activity is also controlled by TIMP-2 and MT1-MMP (13–15), with TIMP-2 inhibiting its activity (17), whereas MT1-MMP removes the propeptide of MMP-2, resulting in its activation (13). In addition, MT1-MMP itself possesses gelatinolytic activity (15). There have been many reports on the enhancement of MMP-2 activity by X-ray irradiation from 2 Gy to 8 Gy (25, 32 33). Wild-Bode *et al.* (32) also reported that sublethal X-ray irradiation of glioma cells increased MMP-2 activity because of down regulation of TIMP-2 and up-regulation of MT1-MMP. Furthermore, administration of *o*-phenantroline, which is one of the MMP inhibitors, significantly inhibited their invasiveness. Our study also showed that MMP-2 activity was increased by X-ray irradiation but inhibited by carbon ion irradiation, indicating that this might relate to the balance of MT1-MMP or TIMP-2 expression levels.

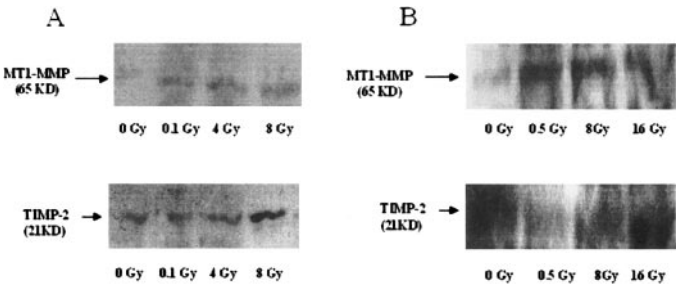

Fig. 5. Effects of irradiation on the expression levels of MT1-MMP and TIMP-2 of ECV304 cells. Lysates from untreated or treated cells were prepared 48 h after irradiation. MT1-MMP and TIMP-2 levels were analyzed by Western blot. *A,* cells were untreated or treated with carbon ion beams. *B,* cells were untreated or treated with X-ray beams.

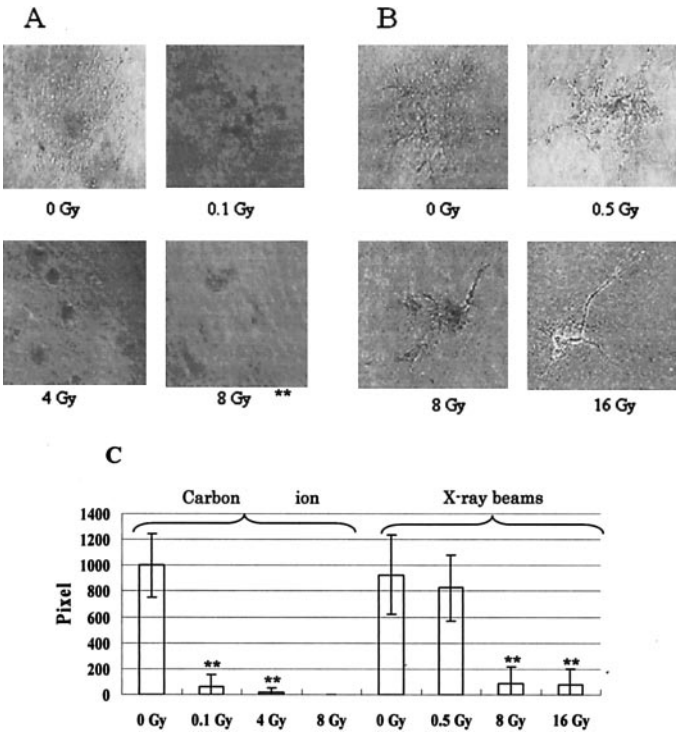

Fig. 6. Effects of irradiation on the formation of capillary-like tube structures assessed in a collagen-embedded culture. *A,* ECV304 cells were untreated or treated with carbon ion beams. *B,* ECV304 cells were untreated or treated with X-ray beams. *C,* quantitation of capillary-like tube structure according to carbon ion or X-ray irradiation by analyzing pixel sizes which correspond to the length and width of capillary tubes as described in "Materials and Methods." Pixel sizes of the lines drawn along tubes are shown on the *vertical axis* and the irradiated doses on the *horizontal axis* ($n = 4$); *bars,* ±SE. **, $P < 0.01$.

Although many studies have shown that MMP-2 played a critical role in angiogenesis, O'Reilly *et al.* (12) demonstrated that MMP-2 was also responsible for the production of angiostatin, which possibly suppresses angiogenesis. Despite inhibition of the MMP-2 activity by carbon ion irradiation, our study showed the destruction of three-dimensional capillary structures. This discrepancy demonstrates that the roles of MMPs can be complicated and can be regulated by other factors. Therefore, more detailed studies on how MMP-2 is acting on *in vitro* angiogenesis by carbon ion or X-ray irradiation will be required.

Radiation therapy inhibits the cell proliferation of vascular endothelial cells *in vitro* (22, 23). Miyamoto *et al.* (34) reported that focal X-ray irradiation of >10 Gy to the corneal region suppressed angiogenesis because of lethal damage to endothelial cells. They also demonstrated that X-ray irradiation of 10 Gy produced transient inhibition, whereas a dose of 20 Gy strongly inhibited corneal angiogenesis. Mauceri *et al.* (35) found that ~1% of HUVEC cells survived

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from X-ray irradiation at 9 Gy. However, our study revealed that surviving cells even after 16 Gy irradiation of X-ray progressed to tube formation in collagen-embedded culture, although most irradiated cells were reduced because of its lethal damage of irradiation. On the other hand, the cell proliferation of ECV304 cells was dramatically reduced by carbon ion irradiation of >4 Gy, whereas there was no difference in cell growth between 0 Gy and 0.1 Gy irradiation with carbon ion beams (data not shown). Surprisingly, however, our findings for collagen-embedded culture showed that even 0.1 Gy of carbon ion irradiation destroyed most of the capillary-like tubes in three-dimensional culture, suggesting that this may not be induced by the inhibition of growth of endothelial cells but by other mechanisms such as inhibition of migration, MMP-2 activity, or down regulation of $\alpha V\beta 3$ of endothelial cells. However, more detailed studies are required to reach the definite conclusion.

In conclusion, our *in vitro* results provide an implication that heavy ion beam therapy could be superior to conventional photon beam therapy in preventive effects on *in vitro* angiogenesis and might inhibit angiogenesis *in vivo*. However, it is important whether heavy ion or X-ray irradiation affects specifically angiogenic microvasculature or both angiogenic and nonangiogenic microvasculatures. We will continue to study whether these results reflect effects on angiogenic microvasculature *in vivo*.

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