Effects of irradiation

on bone invasion of breast cancer cells

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ABSTRACT

Background: Periostin is overexpressed in metastases from bone cancer. Many studies have indicated that periostin plays an important role in bone metastasis. Radiotherapy improves local tumor control, but recent evidence suggests that irradiation of the target tumor can promote tumor invasion and metastasis.

Objective: The purpose of the study was to examine the effects of irradiation with carbon ion or gamma ray on the expression of periostin in breast cancer cells and the cytokine levels of osteoblasts in bone tumor metastases.

Materials and methods: Breast cancer cells (FM3A/R cells) were exposed to carbon ion or gamma ray and then cocultured with non-irradiated osteoblastic MC3T3-E1 cells. Periostin expression in breast cancer cells and the levels of cytokines influencing bone invasion in osteoblastic cells were measured.

Results: Periostin expression increased after irradiation with carbon ion or gamma ray. Carbon ion-irradiated cells expressed less periostin than did gamma ray-irradiated cells. Carbon ion irradiation stimulated low levels of periostin synthesis than gamma ray irradiation. The cytokines influencing bone invasion levels rose in tandem with the increase in periostin level.

Conclusions: Carbon ion irradiation may reduce the production of bone-destroying cytokines and vascularization factors by osteoblasts in the microenvironment of cancer invasion in bone. A combination of carbon ion irradiation and a periostin inhibitor would improve treatment of bone metastatic breast cancer.

Key words: bones; breast cancer; carbon; radiotherapy; periostin

Introduction

Breast cancer has become the principal malignant tumor in females. Many patients die annually from breast cancer metastasis, which triggers advanced skeletal disease, because bone is the preferred site for metastatic dissemination of breast cancer.[1] Metastasis can occur via blood or lymphatic vessels or directly to bone. The biochemical mechanisms involved remain poorly understood in terms of both bone metastasis and bone distraction. Many complications are associated with the development of bone metastases, including pain, hypercalcemia, fracture, and spinal cord compression. Thus, quality of life is severely affected.[2]

Bone metastases are the most frequent indication for palliative radiotherapy in patients with breast cancer. Radiotherapy, alone or in combination with chemotherapy, is widely used. Irradiation may be an effective treatment option in the context of cancer therapy. However, clinical evidence indicates that relapses developing after radiotherapy for treatment of breast cancer are associated with increased metastatic potential and poor prognosis.[3] Tumors growing within previously irradiated mammary tissue tend to be more invasive and to form more metastases in experimental models.[4-6] On the other hand, it has been reported that it is effective for local control and inhibition of metastasis with heavy ion in breast cancer radiotherapy.[7] The National Institute of Radiological Sciences (NIRS) has been carried out the first treatment in April 2013. The final result of treatment outcome will need a longer follow-up period, but effects of carbon radiotherapy on breast cancer are as good as on other cancers.[8,9]

Few reports on cytokine expression in the bone microenvironment after irradiation have appeared. Prevention of skeletal events is one goal of palliative radiotherapy in patients with bone metastases. In the tumor microenvironment, osteopontin (OPN) and other proteins of the extracellular matrix contribute to metastasis, by modulating the maintenance and expansion of both normal and metastatic cancer cells. In particular, the role of receptor activator of nuclear factor kappa-B ligand (RANKL) inducing osteoclasts, and vascular endothelial growth factor (VEGF) inducer of angiogenesis is important in the bone invasion of tumor.[10,11]

Periostin, originally considered an osteoblast-specific factor, is a disulfide-linked 90-kDa secretory protein that functions as a cell adhesion molecule in pre osteoblasts and is involved in osteoblast recruitment, attachment, and spreading.[12] Periostin was originally identified in a mouse osteoblastic library.[13] The name "**periostin**" reflects the expression of the protein in the periosteum and periodontal ligament.[12] The protein is homologous to an insect cell adhesion molecule (fasciclin I) in terms of both sequence and structure, having four repeat domains of 120–160 amino acids. Such proteins often serve as adhesion molecules.[13] Thus, we investigated periostin expression in breast cancer tissue and the clinical relevance thereof.

Periostin plays roles in skeletal development, heart development, disease, and cancer.[13-16] Any possible role for periostin in tumor progression remains unclear, and the associated data are often contradictory. Periostin overexpression has been observed in most cancers, while reports of periostin downregulation are rare and exceptional. Periostin was found to be overexpressed in various types of human cancer.[17-26] Periostin may play an important role in breast cancer bone metastasis.[27,28] Previous reports showed that a novel angiogenic factor expressed in human breast cancer and ovarian cancer significantly enhanced angiogenesis.[10,11,21,29]

In the present study, periostin expression in breast cancer was explored and evidence was obtained that the protein potentially contributed to bone invasion by both breast cancer per se and metastases thereof. In addition, we explored the effects of irradiation on bone invasion using mouse FM3A/R mammary carcinoma cells, and chose to work with these cells to experimentally determine the differences in their responses to carbon ion and gamma ray irradiation. When breast cancer cells were irradiated with different radiation of linear energy transfer (LET) application, the difference response, for example proteins produced in the breast cancer cells after each of irradiation was not due to the cytotoxic effect by LET, but due to difference in quality of LET. In order to verify, we searched for the case of cells whose survival rate after irradiation was not significantly different. As a result, FM3A/R cells met this requirement. The proliferative potentials of FM3A/R cells after carbon ion and gamma ray irradiation [estimated from the median lethal doses (LD50 values)] were approximately equivalent. Moreover, we investigated the mechanisms by which periostin mediated the bone invasion effects of irradiation, and irradiation-induced bone resorption of mammary cancer. Finally, to investigate the mechanisms underlying the effects of irradiation on breast cancer bone invasion and distraction mediated by periostin, we compared the changes in bone resorption factors induced by carbon ion or gamma ray irradiation of breast cancer cells.

The principal purpose of this study was to evaluate the effects of irradiation with carbon ion or gamma ray on the expression of periostin in breast cancer cells and the cytokine levels of osteoblasts in bone tumor metastases.

Materials and methods

Cell culture

FM3A/R breast cancer cells (kindly provided by the Cell Resource Center for Biomedical Research, Institute of Development, Aging, and Cancer, Tohoku University) were grown in RPMI-1640 medium (Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) charcoal-stripped fetal bovine serum (FBS; Gibco, New York, NY, USA), 100 U/mL penicillin, and 100 μg/mL streptomycin (Gibco, Palo Alto, CA, USA) under 5% (v/v) CO_2 in air at 37°C.

MC3T3-E1 osteoblastic cells (derived from the calvarias of newborn C57BL/6 mice) were obtained from the Riken Cell Bank (Tsukuba, Japan), and cultured in DMEM (Invitrogen, Carlsbad, CA, USA) supplemented with 10% (v/v) charcoal-stripped FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37°C under 5% (v/v) CO_2 in air.

Irradiation

FM3A/R cells were cultured into 50 mL cell culture flasks (Falcon 25 cm^2 Flask; Corning Inc. Life Sciences, Tewksbury, MA, USA) grown to about 5 × 10^4 cells/flask (over 2 or 3 days), and then exposed to 5, 10, or 15 Gy of carbon ion or gamma ray irradiation as described previously[30] to evaluate cell proliferation after irradiation. Cells were irradiated in the following method, in order to make the radiation dose of each cell uniform. Carbon ion irradiation was performed using the 290 MeV μm-1, 6-cm Spread-Out Bragg Peak (SOBP) at the Heavy Ion Medical Accelerator in Chiba (HIMAC), NIRS. The estimated LET averaged 40 KeV μm-1 for the proximal SOBP, and the dose rate was 3 Gy min-1. The beam intensity was measured using dose monitors installed in the beam path. Binary filters made of poly (methyl methacrylate) plates (0.5-128 mm thickness) were used to control LET. Gamma ray irradiation was performed at 1 Gy min-1 using a Gamma cell 40 Exactor (Best Theratronics Ltd., Kanata, ON, Canada). Radiation dose and fractionation (5, 10, or 15 Gy)

were decided based on our experience with other breast cancer cells. The survival rate of breast cancer cells FM3A/R decreased after irradiation 10 Gy or more, but periostin production of surviving cells was increased three days after irradiation. According to these previously results, 5, 10, and 15 Gy were irradiated to FM3A/R cells in this experiment.

MTT assay

The relative viable cell numbers of FM3A/R cells were determined by the MTT assay using a Cell Counting Kit-8 (Dojindo Molecular Technologies, Gaithersburg, MD, USA). Briefly, control and irradiated cells were cultured for 24 hours in 90 μL of RPMI-1640 medium at 1000 cells/well, followed by addition of 10 μL of Cell Counting Kit-8 solution to each well. After incubation for 1-4 hours at 37°C in the incubator, the absorbances at 450 nm were measured using a microplate reader (Model 550; Bio-Rad, Hercules, CA, USA). The amount of formazan dye generated by dehydrogenases in the cells was directly proportional to the number of living cells.

Coculture assay

Cocultivation was aimed to investigate the effect of two or more cells on each other. In the present study, irradiated FM3A/R cells, and non-irradiated MC3T3-E1 cells were cocultured in the same dish for the purpose of studying how periostin produced by FM3A/R cells that affected the differentiation and maturation of osteoblastic cells MC3T3-E1. FM3A/R cells were cultured in 50 mL cell culture flasks and then exposed to carbon ion or gamma ray irradiation. After irradiation, FM3A/R cells and medium were immediately transfered into a dish in which non-irradiated MC3T3-E1 cells were cultured approximately 80% confluent. FM3A/R cells were cocultured with non-irradiated MC3T3-E1 osteoblastic cells for 3 days after irradiation. FM3A/R cells floating in the medium were evaluated by RT-PCR assays and MC3T3-E1 cells in dishes were evaluated by western blotting. The floating FM3A/R cells with medium were transferred to centrifuge tube, and after centrifuge, medium was aspirated. FM3A/R cells were used for RT-PCR assays. MC3T3-E1 cells on the bottom of dishes were washed by PBS three times, and used for western blotting.

RT-PCR and real-time PCR

Total RNA was prepared using RNAiso Plus (Takara Bio, Shiga, Japan) according to the manufacturer's instructions. First-strand cDNA was synthesized using a ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan) prior to RT-PCR. Real-time quantitative RT-PCR was performed using DyNamo polymerase (Thermo Fisher, Waltham, MA, USA) and an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). The relative amounts of periostin mRNA were normalized to those of beta-actin mRNA in the same samples. The primers used to amplify the periostin and GAPDH sequences are shown in Table 1. The amplification parameters were as follows: initial denaturation at 94°C for 5 min; 40 cycles of denaturation at 94°C for 30 s, annealing at 62°C for 30 s, and extension at 72°C for 30 s; and final extension at 72°C for 4 min.

Table 1 Primer sequences used for RT-PCR

Gene	Forward Primer	Reverse Primer
GAPDH	CGGAGTCAACGGATTTGGTCGTAT	AGCCTTCTCCATGTTGGTGAAGAC
Periostin	AATGCTGCCCTGGCTATATG	GTAGTGGCTCCCACAATGC

Raft culture

A collagen matrix solution was prepared using seven volumes of type I collagen (Nitta Gelatin, Tokyo, Japan), two volumes of 5× concentrated RPMI-1640 medium, one volume of 10× reconstruction buffer, and FM3A/R cells (1.5×10^5 cells/mL). The final concentration of collagen gel was 0.24% (w/v). Aliquots (2 mL) of the collagen–cancer cell suspension were poured into 60-mm-diameter plastic dishes and allowed to gel for 30 min at 37°C. RPMI-1640 medium (2 mL) was added to each dish and the cells were cultured for 3 days. The collagen–cancer cell suspensions were then fixed in 4% (v/v) paraformaldehyde, dehydrated in a graded series of alcohol baths, embedded in paraffin, and sliced into 4 μm thick sections. The sections were subjected to immunofluorescence staining.

Western blotting

Cells were lysed using cell lysis solution comprised of Pierce RIPA buffer, Halt Protease and Phosphatase Inhibitor Cocktail, and 0.5 M EDTA Solution (all from Thermo Scientific Inc., Rockford, IL, USA). After centrifugation, the supernatants were subjected to SDS-PAGE with 10–20% gel concentrations (ATTO Corp., Tokyo, Japan) and electroblotted onto Immobilon-P transfer membranes (EMD Millipore Corp., Billerica, MA, USA). After blocking with 5% (w/v) skim milk (Wako Pure Chemical Industries, Osaka, Japan), the membranes were incubated with anti-receptor activator of nuclear factor kappa-B ligand (RANKL) and anti-vascular endothelial growth factor (VEGF) (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) primary antibodies and then with horseradish peroxidase-conjugated anti-rabbit IgG as a secondary antibody. Antibody-bound protein bands were visualized using ECL-plus reagent (GE Healthcare, Little Chalfont, UK). An anti-beta-actin monoclonal antibody (013-24553; Wako Pure Chemical Industries) was used to detect beta-actin (internal control).

Recombinant periostin experiments

The expression levels of RANKL and VEGF in MC3T3-E1 osteoblastic cells grown under periostin-enriched conditions were evaluated. Briefly, MC3T3-E1 cells were grown for 3 days in medium supplemented with various concentrations of recombinant periostin. The cells were then lysed and the proteins were extracted. The expression levels

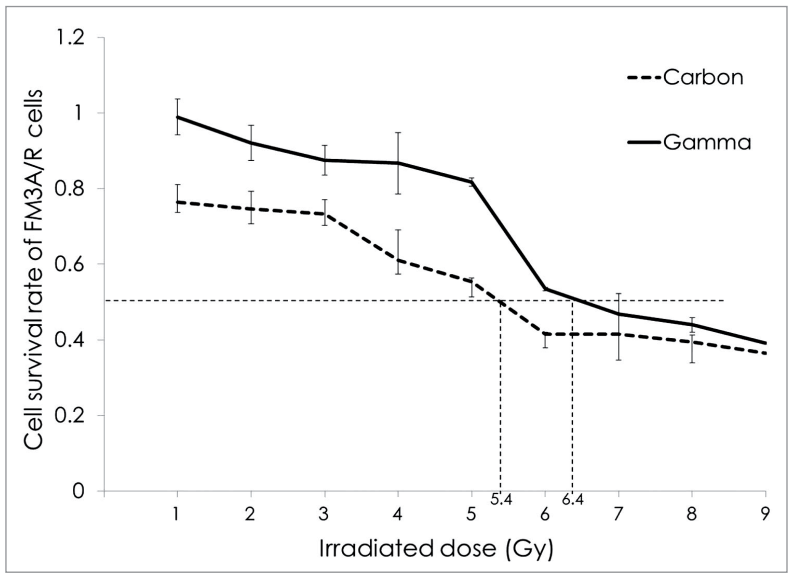

FIGURE 1. Cell survival rates of FM3A/R cells at 24 hours after irradiation were measured by the MTT assay. Cell viability decreased in a dose-dependent manner. LD50 values were estimated from the graphs, and the killing effects of carbon ion irradiation were expressed as ratios to the killing effects of gamma-ray irradiation. Data represent the means ± SD of three experiments.

of RANKL and VEGF in MC3T3-E1 cells were measured by western blotting.

siRNA-induced periostin gene silencing

A periostin siRNA (sc-61325; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) was used to knock down periostin gene expression. FM3A/R cells subjected to carbon ion or gamma ray irradiation were transfected with 50 pmol siRNA in 100 μL of transfection medium, in accordance with the manufacturer's instructions. After 8 h, the FM3A/R cells were subjected to the coculture assays described above. A scrambled siRNA sequence lacking significant homology to any rat, mouse, or human gene sequence was used as a control.

Results

FM3A/R cells were treated with various doses of carbon ion or gamma ray irradiation and evaluated for their cell proliferation. Cellular replication was decreased after both carbon ion and gamma ray irradiation in a dose-dependent manner. The proliferative potentials of the cells (Figure 1) were estimated by reference to the LD50 values of radiation, and were approximately 1.18 after both types of irradiation. FM3A/R cells were used for these experiments because the cell survival rates were nearly equal after exposure to the two types of irradiation.

FM3A/R cells were exposed to 5, 10, or 15 Gy of carbon ion or gamma ray irradiation, and their periostin expression levels were measured by RT-PCR. Periostin expression was decreased immediately after irradiation, but recovered after 3 days. On day 3 post irradiation, higher doses of radiation were associated with rapid increases in periostin expression. Besides, periostin expression in irradiated cells increased to higher levels than those in non-irradiated cells. Moreover, cells subjected to gamma ray irradiation expressed higher levels of periostin than cells subjected to carbon ion irradiation (Figure 2).

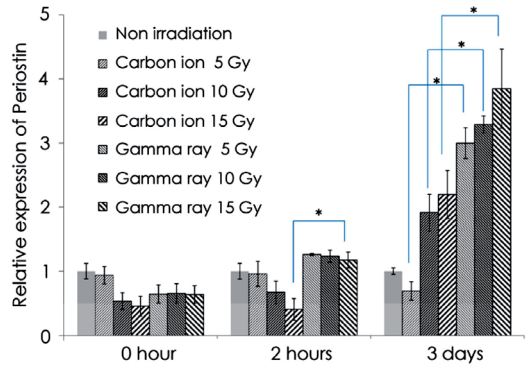

FIGURE 2. FM3A/R cells were exposed to 5, 10, or 15 Gy of carbon ion or gamma ray irradiation. Total RNA was extracted and their periostin mRNA levels were measured by RT-PCR at 0 hour, 2 hours, and 3 days after irradiation. Significant increases in these levels were evident in gamma ray-irradiated cells by day 3 post irradiation. Expression of mRNA was represented by ratio. The non-irradiated cells are shown as 1 (paired t-test, *P < 0.05). Data represent the means ± SD of three experiments.

The periostin levels were increased in irradiated FM3A/R cells, as shown in Figure 3. FM3A/R cells grew in a collagen gel, and formed clumps. Sections of irradiated cells were stained more strongly for

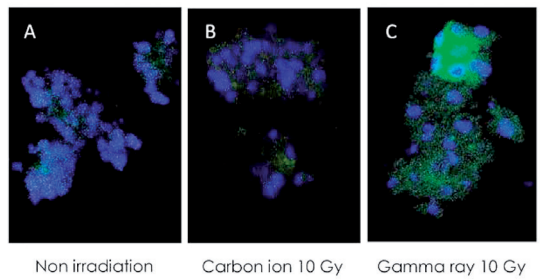

FIGURE 3. Irradiated FM3A/R cells in raft cultures were assessed for periostin expression. (A) Non irradiation. (B) Carbon ion irradiation (10 Gy). (C) Gamma ray irradiation (10 Gy). Immunohistochemically, expression of periostin (green) in the cytoplasm was observed surrounding the nucleus (blue) in both irradiated and non-irradiated FM3A/R cells. Gamma ray-irradiated cells expressed high levels of periostin.

periostin than sections of non-irradiated cells. In particular, gamma ray irradiation significantly increased periostin expression, as evidenced by the density of immunofluorescence staining.

The periostin levels in irradiated FM3A/R breast cancer cells were clearly higher than those in non-irradiated cells. Next, the effects of periostin on MC3T3-E1 osteoblastic cells were examined. For this, MC3T3-E1 osteoblastic cells cocultured with carbon ion- or gamma ray-irradiated FM3A/R breast cancer cells were compared. The expression levels of RANKL and VEGF in MC3T3-E1 cells cocultured with carbon ion-irradiated FM3A/R cells were lower than those in MC3T3-E1 cells cocultured with gamma-irradiated FM3A/R cells. The RANKL and VEGF protein levels in cocultured MC3T3-E1 cells were measured by western blotting. The levels of RANKL (bone invasive cytokine) in MC3T3-E1 cells were significantly increased upon coculture with gamma-irradiated FM3A/R cells (Figure 4).

Periostin produced by irradiated FM3A/R breast cancer cells may enhance the expression of RANKL and VEGF in MC3T3-E1 osteoblastic cells cocultured with the breast cancer cells. The expression levels of RANKL and VEGF after addition of recombinant periostin (as a supplement) to osteoblastic culture medium were measured (Figure 5).

The levels of both RANKL and VEGF increased with the recombinant periostin level, and the levels were similar to those seen upon coculture with irradiated FM3A/R breast cancer cells. The RANKL and VEGF levels in MC3T3-E1 osteoblastic cells cocultured with irradiated FM3A/R cells were also measured after siRNA-mediated suppression of periostin synthesis. The siRNA-mediated suppression inhibited the expression of RANKL and VEGF in MC3T3-E1 cells (Figure 6).

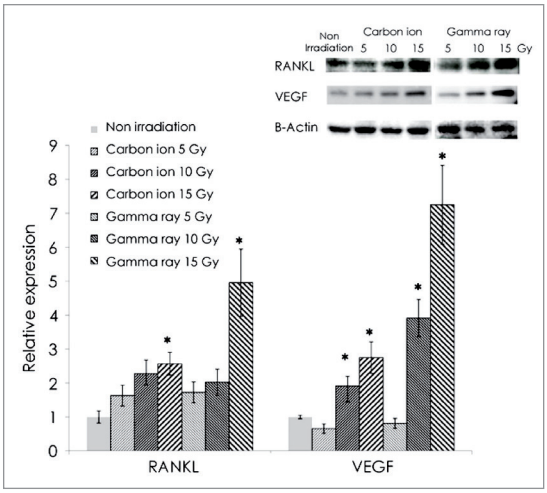

FIGURE 4. Immediately after carbon ion or gamma ray irradiation, FM3A/R breast cancer cells were cocultured with non-irradiated MC3T3-E1 osteoblastic cells. Total protein was extracted from the adherent MC3T3-E1 cells. The RANKL and VEGF protein levels were measured by western blotting. The levels of RANKL and VEGF in cocultured MC3T3-E1 cells increased with the periostin levels in irradiated FM3A/R cells. The increases were significant upon coculture with FM3A/R cells exposed to higher doses of irradiation. Expression of target protein was represented by ratio. The non-irradiated samples are shown as 1 (paired t-test, *P < 0.05). Data represent the means ± SD of three experiments.

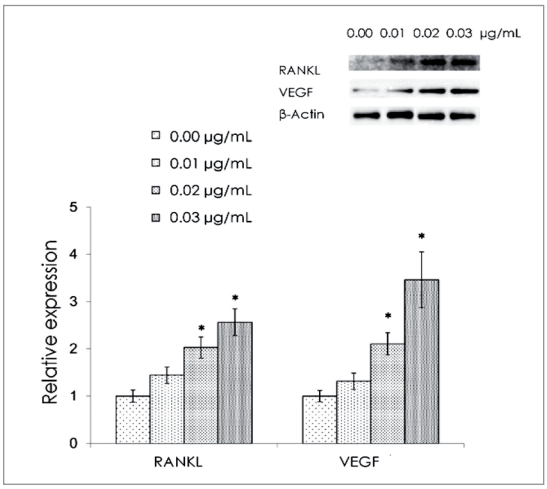

FIGURE 5. MC3T3-E1 cells were cultured in medium supplemented with recombinant periostin for 3 days, and then measured for their RANKL and VEGF levels by western blotting. The 0.00 µg/mL periostin-supplemented cell group is shown as 1 (paired t-test, *P < 0.05). Data represent the means ± SD of three experiments.

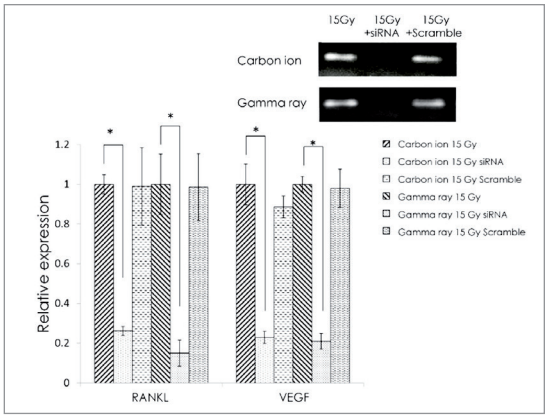

FIGURE 6. Irradiated FM3A/R cells (15 Gy) were cultured with a siRNA targeting periostin and a decrease in the periostin levels was confirmed by RT-PCR (upper right). Periostin expression was not suppressed in cells cultured with a control scrambled siRNA. The RANKL and VEGF levels in MC3T3-E1 cells cocultured with transfected and irradiated FM3A/R cells expressing reduced levels of periostin revealed suppressed expression of RANKL and VEGF. Expression of target protein was represented by ratio. The non-transfected cell groups are shown as 1 (paired t-test, *P < 0.05). Data represent the means ± SD of three experiments.

Discussion

The effects of irradiation on cancer cell metastasis have been poorly studied.[31,32] It has been suggested that ionizing radiation increases cancer cell escape into the lymphatic and vascular channels. However, few data are available. Irradiated tissue may be more metastatic than non-irradiated tissue. Carbon ion irradiation is effective for both killing cells and suppressing metastatic potential. An understanding of the effects of carbon ion irradiation on the metastatic potential of bone metastatic cancer cells might improve the clinical outcomes of cancer radiotherapy.

FM3A/R mouse breast cancer cells that can invade bone were studied, because these cells responded similarly to carbon ion and gamma ray irradiation, as assessed by reference to the LD50 values.

In general, high LET radiation has high cells killing effect. The survival rate of cells after carbon ion irradiation is lower than at the same dose of gamma ray irradiation markedly. It is difficult to differentiate the difference in the cells response after carbon ion or gamma ray irradiation due to the difference of LET or the cell viability of irradiation. For this reason, we searched cells that did not differ greatly in the cell survival rate after irradiation due to the difference of LET. Therefore, we used FM3A/R cells in this study.

Periostin expression in FM3A/R cells was assessed by RT-PCR, commencing immediately after irradiation and continuing over the next 3 days. Periostin expression was decreased immediately after irradiation, but increased 2 hours later. Irradiation exerted a clear antiproliferative effect. In addition, irradiation stimulated cancer cells to produce periostin in a dose-dependent manner, being particularly evident in gamma ray-irradiated cells. Bone invasion by cancer is caused by osteoclasts induced by cancer cells and osteoblasts. The osteoclast-inducing factor RANKL, and VEGF, are produced by osteoblasts.[10,33] Both RANKL and VEGF levels were increased upon coculture with irradiated cells. MC3T3-E1 osteoblastic cells cocultured with FM3A/R breast cancer cells exposed to higher doses of irradiation expressed RANKL and VEGF strongly, while MC3T3-E1 cells cocultured with gamma ray-irradiated FM3A/R cells expressed both cytokines to greater extents than MC3T3-E1 cells cocultured with carbon ion-irradiated FM3A/R cells.

It was hypothesized that periostin triggered the observed increases in RANKL and VEGF levels. Thus, MC3T3-E1 osteoblastic cells were cultured in medium supplemented with recombinant periostin for 3 days, and measured for their RANKL and VEGF levels. Both cytokines increased with the periostin concentration, suggesting that cancer invasiveness was enhanced by periostin. It is possible that irradiation of breast

cancer cells enhances periostin expression, in turn accelerating bone metastasis by cells that survive irradiation. Gamma ray irradiation possibly induces more periostin synthesis than carbon ion irradiation.

The effects of a reduction in the periostin level were also evaluated. MC3T3-E1 osteoblastic cells were cocultured with irradiated FM3A/R breast cancer cells previously cultured in medium with a periostin siRNA, and measured for their expression levels of RANKL and VEGF. Both levels were decreased, compared with cells cultured with the scrambled siRNA control. It is thus possible that bone absorption and vascularization are enhanced by periostin, in turn promoting cancer cell metastasis and tumor invasion.

Conclusion

Carbon ion irradiation was slightly superior to gamma ray irradiation in terms of killing FM3A/R breast cancer cells capable of invading bone, but carbon ion irradiation stimulated lower levels of periostin synthesis than gamma ray irradiation. Thus, carbon ion irradiation may reduce the production of bone-destroying cytokines and vascularization factors by osteoblasts in the microenvironment of cancer invasion in bone. Therefore, carbon ion irradiation may be a more effective therapy for cancers capable of invading bone, by suppressing the development of distant metastases. However, metastasis remains possible after carbon ion irradiation. To suppress the expression of periostin in breast cancer cells after irradiation, combined treatments (carbon ion irradiation and administration of a periostin inhibitor) may be indicated.

Acknowledgments

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