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Relative biological effectiveness in canine osteosarcoma cells irradiated with accelerated charged particles

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Abstract. Heavy ions, characterized by high linear energy transfer (LET) radiation, have advantages compared with low LET protons and photons in their biological effects. The application of heavy ions within veterinary clinics requires additional background information to determine heavy ion efficacy. In the present study, comparison of the cell-killing effects of photons, protons and heavy ions was investigated in canine osteosarcoma (OSA) cells *in vitro*. A total of four canine OSA cell lines with various radiosensitivities were irradiated with ^{137}Cs gamma-rays, monoenergetic proton beams, 50 keV/μm carbon ion spread out Bragg peak beams and 200 keV/μm iron ion monoenergetic beams. Clonogenic survival was examined using colony-forming as says, and relative biological effectiveness (RBE) values were calculated relative to gamma-rays using the D_{10} value, which is determined as the dose (Gy) resulting in 10% survival. For proton irradiation, the RBE values for all four cell lines were 1.0-1.1. For all four cell lines, exposure to carbon ions yielded a decreased cell survival compared with gamma-rays, with the RBE values ranging from 1.56-2.10. Iron ions yielded the lowest cell survival among tested radiation types, with RBE values ranging from 3.51-3.69 observed in the three radioresistant cell lines. The radiosensitive cell line investigated demonstrated similar cell survival for carbon and iron ion irradiation. The results of the present study suggest that heavy ions are more effective for killing radioresistant canine OSA cells when compared with gamma-rays and protons. This markedly increased efficiency of cell killing is an attractive reason for utilizing heavy ions for radioresistant canine OSA.

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Introduction

Osteosarcoma (OSA) is the most common malignant bone cancer in dogs, representing 85% of canine skeletal neoplasms (1). OSA is a highly aggressive and painful tumor that occurs primarily in the appendicular skeleton of large and giant breed dogs (1). Treatment of OSA is difficult due to its high metastasis rate and aggressive local behavior (2). The current standard treatment for OSA in dogs is amputation or limb-sparing surgery combined with chemotherapy (2). Amputation or limb-sparing surgery is not always the choice of treatment for dogs with OSA due to neurologic or orthopedic disease (1). Alternative treatments to suppress tumor growth and alleviate the pain of the primary tumor would have significant clinical relevance in these cases. Coarsely fractionated, or 'palliative' radiation therapy using several fractioned doses (for example, 8-10 Gy per fraction) may be used for palliation of pain, but is not able to achieve local tumor control (3-5). High dose per fraction radiation therapy has been described as a method for local tumor control using stereotactic radiosurgery, particularly when combined with chemotherapy (5,6). Stereotactic radiosurgery is able to deliver a single very high dose of radiation (for example, 30 Gy) using multiple beams with a linear accelerator to a designated target, while sparing the surrounding tissues (6).

Charged particle radiation therapy, including proton and heavy ion therapy, has gained interest in human radiation oncology as a novel therapeutic modality (7). Clinical application of protons and carbon ions for human cancer treatment has been expanding and has achieved significantly improved clinical outcomes for a number of tumor types, including OSA in non-resectable tumors (8). Unlike conventional photon radiation, protons and carbon ions may be manipulated to

release the majority of their energy only when they reach their target (7,9). This property is known as the Bragg peak. Particle radiation therapy offers the possibility of a significantly increased dose of ionizing radiation to the tumor with very little dose deposited in the normal tissue (7-9). Particle radiation therapy has the potential to be considered as an alternative modality for the treatment of canine and human OSA in the future. Carbon ions have increased ionization density [high linear energy transfer (LET) properties], and heavy ions differ from protons or photons with low LET due to their radiobiological properties. A number of *in vitro* studies have demonstrated that high LET radiation is more effective than low LET radiation in terms of cell-killing effects (10-13). Additionally, the extent of cell killing by heavy ions is not cell cycle dependent and is not decreased due to the presence of hypoxia (14-16). In human oncology, the relative biological effectiveness (RBE) is used to describe the increase in effectiveness of particle radiation (10,12). RBE is defined as the ratio of doses of photons and charged particles inducing the same biological endpoints, including cell killing, mutation and cytogenetic aberrations. The RBE values of cell killing for heavy ions generally increases up to a LET near 200 keV/μm and decreases afterwards (17,18).

Particle radiation therapy may be expensive to implement, and the application of heavy ions within veterinary clinics requires additional background information to determine its effectiveness. To the best of our knowledge, there are only limited reports of the use of protons for treating dogs with brain tumors and currently there have been no reports of using heavy ions to treat tumors in veterinary medicine (19,20). RBE values of 1.96-2.50 in canine squamous cell carcinoma, fibrosarcoma and hemangiopericytoma cell lines for carbon ions (LET=108 keV/μm) have been previously reported (21). Our previous study described canine OSA cell lines that were either radioresistant or highly radiosensitive to photon radiation, along with their basic cellular characteristics (22). In the present study, an *in vitro* comparison of the cell-killing effects of photons, protons and heavy ions on canine OSA cells was performed. For heavy ion irradiation, SOBP (spread out Bragg peak) carbon ions (LET at 50 keV/μm), which are used in radiotherapy, and iron ions (LET at 200 keV/μm), as above are expected to have maximum biological effects of heavy ions (23).

Materials and methods

Cell culture. The canine OSA cell lines Abrams, D17, Grey and Moresco were a gift from the Animal Cancer Center of Colorado State University (Fort Collins, CO, USA) (22,24). Cells were grown in minimal essential media (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) supplemented with 10% (v/v) fetal bovine serum (FBS; Sigma-Aldrich, St. Louis, MO, USA) and 1% (v/v) Pen/Strep and fungizone solution (Invitrogen; Thermo Fisher Scientific, Inc.), and they were maintained in a tissue culture incubator at 37˚C in a 100% humidified atmosphere of 5% CO_2. The cell doubling times of these cells were 19 h for Abrams, 22 h for D17, 18 h for Grey and 22 h for Moresco (22).

Radiation conditions. Particle-based irradiation experiments were performed at the National Institute of Radiological Sciences (NIRS) in Chiba, Japan. For heavy ion exposure, accelerated ions were irradiated using the Heavy Ion Medical Accelerator in Chiba (HIMAC; Chiba, Japan) at room temperature. The details concerning the beam characteristics of the particle radiation, biological irradiation procedures and dosimetry have been described previously (12,25,26). Accelerated monoenergetic iron ions have 500 MeV/nucleon of initial energy and 200 keV/μm of LET at the irradiated position. Carbon ions were accelerated at 290 MeV/nucleon of initial energy and spread out with a ridge filter for 6 cm width of SOBP. The monolayer cell culture was irradiated at the center (50 keV/μm of average LET) within the SOBP at a distance of 119 mm from the entrance (27). Monoenergetic protons that were accelerated to 70 MeV using the NIRS-930 cyclotron have a LET value of 1.0 at the irradiated position. Dose rates for heavy ions and protons were set at 1 and 5 Gy/min respectively. Gamma-ray irradiations were performed at the Colorado State University (Fort Collins, CO, USA) with ^{137}Cs gamma-rays delivered at a dose rate of ~2.5 Gy/min at room temperature (using a J.L. Shepherd Model Mark I-68, nominal 6000 Ci ^{137}Cs irradiator).

Cell survival assays. Exponentially growing cells cultured in T12.5 flasks (BD Biosciences, Franklin Lakes, NJ, USA) were irradiated, then trypsinized and plated onto 100 mm cell culture dishes at an density of 100 colonies. Following incubation for 7-10 days to allow colony formation, surviving colonies were rinsed with 0.9% NaCl, fixed with 100% ethanol and stained by 0.1% crystal violet. Each colony consisting of >50 cells was scored as a survivor. At least three independent experiments were performed.

RBE was calculated, which is defined as the ratio of dose of photons and charged particles inducing identical biological effects, based on D_{10} values. The D_{10} values, which represent doses required to achieve 10% survival, were obtained from each survival curve using Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA).

Statistical analysis. Data were analyzed using Prism 5 software. Data are presented as the mean ± standard error. Differences with a $P<0.05$ were considered statistically significant. Statistical comparison of mean values in the RBE was performed using unpaired two tailed t-test.

Results

The present study used canine OSA cell lines with various radiation sensitivities as reported previously (22). Abrams, D17 and Moresco were fairly resistant to gamma-rays (D_{10}=7.12-9.33 Gy), while Grey was relatively radiosensitive (D_{10}=2.72 Gy). Fig. 1 shows the dose-response curves for cell-killing effect on the four canine OSA cell lines irradiated with various radiation sources. It was observed that the clinically relevant carbon ion beams (LET at 50 keV/μm) decreased cell survival fractions of the four canine OSA cell lines compared with gamma-rays ($P<0.05$). Iron ion beams decreased cell survival more than carbon ions in the three radioresistant cell lines (Abrams, D17 and Moresco). However, the radiosensitive cell line Grey demonstrated similar cell survival for both carbon and iron ion irradiation. The proton

Table I. Relative biological effectiveness of particle radiation types in canine osteosarcoma cell lines.

	γ-rays, 0.2 keV/μm	Proton, 1 keV/μm		Carbon SOBP, 50 keV/μm		Iron, 200 keV/μm	
Cell lines	D_{10}, Gy	D_{10}, Gy	RBE	D_{10}, Gy	RBE	D_{10}, Gy	RBE
Abrams	7.12	3.96	1.80	3.81	1.87	2.01	3.55
D17	9.33	7.26	1.29	4.44	2.10	2.53	3.69
Grey	2.72	3.02	0.90	1.55	1.75	1.23	2.22
Moresco	7.24	6.68	1.08	4.64	1.56	2.62	3.51

D_{10} values represent doses required to achieve 10% survival. RBE was obtained from (D_{10} of γ-rays)/(D_{10} of particle irradiation). SOBP, spread out Bragg peak beams; RBE, relative biological effectiveness.

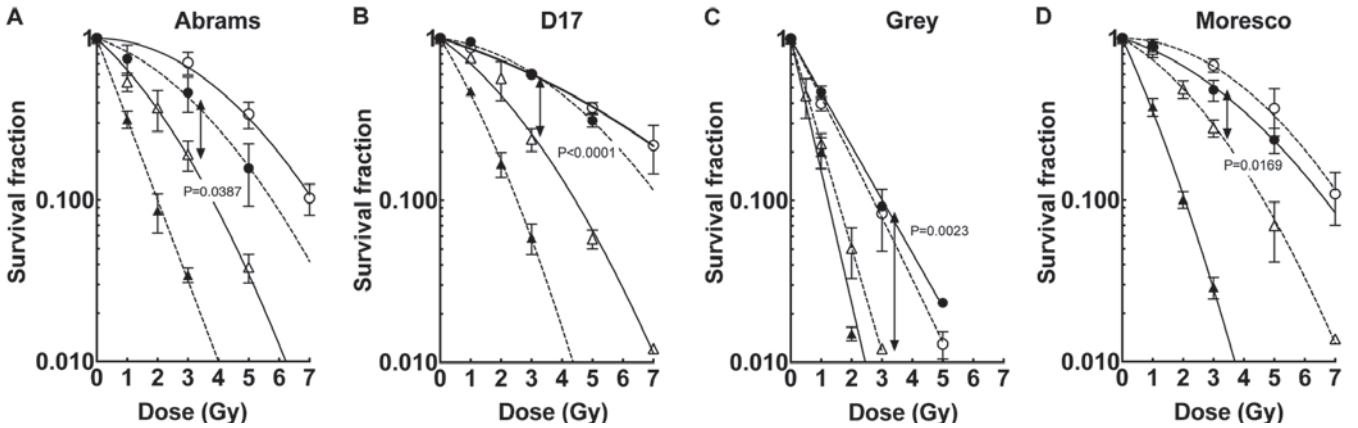

Figure 1. Survival curves for gamma-rays, proton and carbon ion irradiation in four canine osteosarcoma cell lines. White circle indicates gamma-rays, LET 0.2 keV/μm; black circle indicates protons, LET 1 keV/μm; white triangle indicates carbon ions, LET 50 keV/μm; and black triangle indicates iron ions LET 200 keV/μm. Experiments were performed at least three times and data are presented as the mean ± standard error. LET, linear energy transfer.

cell survival curves demonstrated similar profiles to those of gamma-rays for the four cell lines. In order to describe the increased effects of particle radiation, RBE was calculated based on the D_{10} values relative to gamma-rays (Fig. 2 and Table I). The RBE values ranged from 0.90-1.26 for protons, 1.56-2.10 for carbon-ions and 2.22-3.69 for iron ions among the cell lines. The RBEs for iron ions in the radiosensitive Grey cell line (2.22) was reduced compared with those in the three radioresistant cell lines.

Discussion

The present study indicated that heavy ions may be a superior method to induce canine OSA cell killing *in vitro* compared with protons and gamma-rays, based on using four cell lines with various radiosensitivities. The results of the present study were consistent with a previous canine cell line study (21). Additionally, several other previous studies using human and rodent cells have demonstrated that high LET heavy ions are more effective for cell killing at identical physical absorbed doses as low LET radiation types (10-12,21,26). In general, a large number of complex clusters of DNA damage generated by high LET radiation would be more difficult to repair, resulting in more severe biological damage than that induced by low LET radiation (28,29).

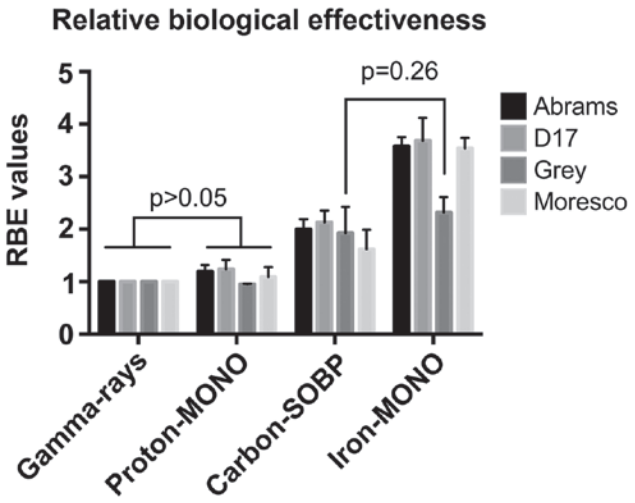

Figure 2. RBE values calculated from dose to get D10 survival fractions. The RBE values for protons (LET 1 keV/μm), carbon ions (LET 50 keV/μm) and iron ions (LET 200 keV/μm) were obtained for each cell line relative to gamma-rays on each cell line. RBE, relative biological effectiveness; LET, linear energy transfer; SOBP, spread out Bragg peak beams; MONO, monoenergetic.

In the present study, the RBE values for the human clinical setting carbon ions ranged from 1.56-2.10 using D_{10} doses relative to that of gamma-rays. The RBE values of canine

OSA cells for the carbon ions were close to the previously published values: ~1.5 for human salivary gland tumor origin HSG cells and 1.8 for HeLa cells, using identical carbon-ion beams (30). For a proton beam, which is another type of particle radiation with low LET, the cell killing effects did not differ substantially from gamma-rays, providing the RBE values of almost 1.0 for all four cell lines. In human oncology, the RBE of protons has been reported to exhibit experimental variations, but in general a constant RBE of either 1.0 or 1.1 is estimated for clinical applications (31-34). However not only LET, but also DNA repair capacity and cellular radiosensitivity affect RBE values (7,26). The results of the present study suggest that the effectiveness of particle radiation on human and canine tumor cell killing is likely comparable.

Furthermore, the present results with regard to iron ion radiation clearly revealed that heavy ions with high LET (200 keV/μm) better enhanced the cell killing effects in radioresistant canine OSA cells compared with 50 keV/μm carbon ions. It should be noted that this is consistent with previous human and rodent studies, which observed the peak RBE is approximately LET 200 keV/μm (17,18). In the case of the radiosensitive cell line Grey, carbon and iron ion radiation demonstrated more similar cell killing effects and smaller RBE values compared to the radioresistant canine OSA cells. The response of Grey to high LET was similar to DNA-repair deficient cell lines in previous human and rodent studies (26,35). Previous studies have suggested that high LET is effective to cells with normal cellular DNA repair capacity (36,37). Furthermore, in a recent investigation, a variable response to radiation was detected clinically in dogs affected by OSA (38). Although it was beyond the scope of the present study, investigation into the DNA repair deficiencies of the radiosensitive OSA cell line may provide an insight into whether tumor cell radiosensitivity can be used as an indicator of clinical response to radiation therapy.

Canine OSA cells are considered to be resistant to conventional radiotherapy (1). In addition to improved dose distribution conferred by particle irradiation, a markedly increased efficiency of cell killing is another attractive reason for investigating heavy ions (15). Furthermore, hypoxic cells naturally occur within cancerous tissues. Hypoxic cells are resistant to low-LET photons, but may be effectively killed by high-LET heavy ions (14,39). According to previous reports, it has been suggested that heavy ions may suppress the metastatic capabilities of cancer cells; thus, the effects of heavy ions on the control of lung metastasis of canine OSA should be investigated for clinical application (40,41). Additional investigations are required to determine whether heavy ion radiotherapy is able to confer clinical advantages over photon irradiation in veterinary clinics, including in the treatment of dogs with OSA.

In conclusion, the results of the present study provide basic insights into the application of heavy ions in veterinary clinics. With the use of radioresistant and radiosensitive canine OSA cells, the present study demonstrated that high LET charged particles, particularly iron ions, are effective at killing canine OSA cells independent of gamma-ray radiosensitivity. Radioresistant and radiosensitive cells exhibited significantly higher RBE values for carbon and iron ion radiation ($P<0.05$; Fig. 2). These findings support the future investigation of heavy ion application within veterinary clinics to study their effectiveness compared with currently available radiation therapy.

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