Development of an intramuscular xenograft model of canine osteosarcoma in mice for evaluation of the effects of radiation therapy

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Objective—To develop an IM xenograft model of canine osteosarcoma in mice for the purpose of evaluating effects of radiation therapy on tumors.

Animals—27 athymic nude mice.

Procedures—Mice were randomly assigned to 1 of 3 groups of 9 mice each: no treatment (control group), radiation at 10 Gy, or radiation at 15 Gy. Each mouse received $5 \times 10^5$ highly metastasizing parent osteosarcoma cells injected into the left gastrocnemius muscle. Maximum tumor diameter was determined with a metric circles template to generate a tumor growth curve. Conscious mice were restrained in customized plastic jigs allowing local tumor irradiation. The behavior and development of the tumor xenograft were assessed via evaluations of the interval required for tumor-bearing limbs to reach diameters of 8 and 13 mm, extent of tumor vasculature, histomorphology of tumors, degree of tumor necrosis, and existence of pulmonary metastasis and clinical disease in affected mice.

Results—Tumor-bearing limbs grew to a diameter of 8 mm (0.2-g tumor mass) in a mean ± SEM interval of 7 ± 0.2 days in all mice. Interval to grow from 8 to 13 mm was significantly prolonged for both radiation therapy groups, compared with that of the control group. Histologic evaluation revealed the induced tumors were highly vascular and had characteristics consistent with those of osteosarcoma. Pulmonary metastasis was not detected, and there was no significant difference in percentage of tumor necrosis between groups.

Conclusions and Clinical Relevance—A reliable, repeatable, and easily produced IM xenograft model was developed for in vivo assessment of canine osteosarcoma. (Am J Vet Res 2009;70:127–133)
and to determine percentages of tumor necrosis that result from specific doses of radiation. Our hypotheses were that 1 radiation dose of 10 or 15 Gy would significantly delay tumor growth and that radiation therapy would induce dose-dependent tumor necrosis. The usefulness of such a model is that it could be used to evaluate the effectiveness of various radiation therapy protocols and the effects of radiopotentiating Agents. Furthermore, the IM xenograft model could serve as a translational model for human osteosarcoma, given the biologic and histologic similarities between canine and human osteosarcoma.17

Materials and Methods

Animals—Twenty-seven 5-week-old athymic mice were used. All mice received sterilized food and water ad libitum and were housed in a specific pathogen-free barrier facility with 12-hour light and dark cycles. All experiments were performed at the University of Florida in accordance with institutionally approved guidelines for animal welfare.

Cell culture—Canine highly metastasizing parent osteosarcoma cells were obtained.8 Growth medium for HMPOS cells was formulated by supplementing RPMI 1640 solution with 10% heat-inactivated fetal calf serum,1 1% antimicrobials (penicillin [10,000 U/mL] and streptomycin [10,000 µg/mL]),1 1% l-glutamine,1 2% vitamin solution,2 and 1% nonessential amino acids.3 Cells (2 × 106) were seeded into 75-cm2 flasks and maintained at 37°C in 5% carbon dioxide and 95% room air. Cells were grown to confluence (3-day passage time), washed with Hanks’ balanced salt solution (pH, 7.4), detached from their plates with 0.25% trypsin, and counted with a hemacytometer. Cells were precipitated and resuspended in PBS solution (pH, 7.4) to a concentration of 5 × 106 cells/0.02 mL (25 × 106 cells/mL) for IM injection. This suspension was transported on ice to the animal housing facility.

Induction and measurement of tumors—For the xenograft procedure, mice were handled and restrained separately such that the left pelvic limb (recipient for xenograft) of each mouse was immobilized over the handler’s left index finder, and the tail was immobilized between the handler’s left index and middle fingers. Xenografts were initiated via IM injection of 5 × 106 HMPOS cells suspended in PBS solution (total injection volume, 0.02 mL) into the left gastrocnemius muscle. Mice were weighed at least every other day and evaluated daily for primary tumor growth at the site of injection, changes in behavior, and general appearance.

When tumors began to appear, tumor-bearing limbs were measured and examined daily for morphologic changes such as skin ulceration. Maximum tumor diameter was determined with a metric circles template by passing the tumor-bearing limb through the appropriate circle until minimal skin-to-template contact was detected. Tumor diameter measurements were then converted to tumor volume and weight by means of the following formula: tumor volume = (1/6)πd³ – 100, in which d is the diameter of the hole and 100 represents a volume correction factor for a mouse limb without a tumor.18 Tumor volume in millimeters3 was used to approximate tumor weight (100 mm3 = approx 0.1 g). Data from daily measurements were used to generate a tumor growth curve.

When maximum limb diameter reached 8 mm (tumor mass, 0.2 g), mice were randomly assigned to 1 of 3 treatment groups: no treatment (control group; n = 9 mice), radiation of tumor-bearing limb at 10 Gy (9), and radiation of tumor-bearing limb at 15 Gy (9).

Radiation therapy—Tumors were considered suitable to commence radiation therapy when the injected area of the limb reached a maximum diameter of 7.5 to 8.5 mm (corresponding to a tumor weight of approximately 0.2 g). For radiation treatments, all mice were transported simultaneously to a holding room outside the room containing the linear accelerator and remained in their cages until treatments were initiated. Conscious mice were restrained in customized plastic jigs, and the tumor-bearing limb was extended through an opening in the side of the jig. Tumors were irradiated by use of a 6-MV linear accelerator at a rate of 4 Gy/min with an 8-mm-thick gelatin radiation bolus. Tumor radiation dose was calculated by hand along the central axis of the x-ray beam, and dose rate was monitored by means of the linear accelerator.

Necropsy and histologic examination—All mice were euthanized with CO2 followed by confirmatory thoracotomy when their tumor-bearing limb reached a maximum diameter of 13 mm (tumor weight, approx 1.0 g) at the level of the gastrocnemius muscle or earlier when quality of life was deemed compromised. After euthanasia, a complete necropsy was performed. The primary tumor was dissected with the limb in situ, bisected at its greatest diameter, and fixed in neutral-buffered 10% formalin. The respiratory tract (larynx to lungs), heart, and mediastinal fat were dissected free, and the lungs were inflated with neutral-buffered 10% formalin. After fixation in formalin for 48 hours, all tissues were transferred into 70% alcohol. The primary tumor was embedded in paraffin, and 5-µm-thick tumor sections were cut. The tracheobronchial tree and attached pulmonary parenchyma were also embedded in paraffin, and multiple, longitudinal, 5-µm-thick sections parallel to the trachea were obtained to include both left and right lung lobes. All sections were routinely stained with H&E for microscopic examination.

Immunohistochemistry protocol—to evaluate histomorphology of tumors, primary tumors were embedded in paraffin. After tissue processing and embedding, tissue blocks were cut into 5-µm-thick sections and placed in a 40°C to 45°C flotation bath of distilled water. Sections deemed to be of best quality were affixed to adhesive-treated microscope slides. Slides were then deparaffinized in xylene, rinsed in Tris buffer, and incubated in 3% hydrogen peroxide for 10 minutes to stop endogenous peroxidase activity. Heat-induced epitope retrieval was performed by placing slides in citrate buffer in a pressure cooker for 20 minutes. After slides were cooled and rinsed, rabbit polyclonal antibody CD31 (1:100 dilution), a marker of angiogenesis, was applied and the slides were incubated for 30 minutes. The slides were
again rinsed, and biotinylated goat anti-rabbit antibody was applied. Streptavidin peroxidase reagent was added, and chromogen (3,3-diaminobenzidine) was applied. The slides were counterstained with hematoxylin, acetic acid reagent, and bluing reagent. Specimens of human tonsil and mouse heart and kidney were used as positive control tissues.

Tumor measurements—The behavior and development of the IM HMPOS tumor xenograft were evaluated by means of several indices. The number of days required for the tumor-bearing limbs to grow in diameter to 8 mm and from 8 to 13 mm was calculated for each mouse. To evaluate the extent of angiogenesis in tumors in control mice, the areas of highest neovascularization were detected by scanning the tumor sections under low-power (40X and 100X) magnification, and the areas with the highest numbers of discrete microvessels staining positive for CD31 were identified. These areas were subjectively graded on a scale of 1 to 4 (1 = 1 to 2 vascular profiles/400X field; 2 = 3 to 4 vascular profiles/400X field; 3 = 5 to 6 vascular profiles/400X field; and 4 = > 7 vascular profiles/400X field). Each vessel evident within a light-microscope field at 200X magnification was counted, and each count was expressed as the highest number of microvessels identified in a tumor specimen within any field.

Staining of the same tissue sections with H&E was carried out to compare the distribution of tumor vasculature or necrosis within the tumor. During examination of slides for tumor histomorphology, digital images of tumor sections (maximum tumor diameter) were captured with a microscope with an attached 10-megapixel camera and processed with commercial software. Percentage of tumor necrosis was calculated by dividing the area of tumor necrosis by the total area within a light-microscope field at 200X magnification. Each vessel evident within a light-microscope field at 200X magnification was counted, and each count was expressed as the highest number of microvessels identified in a tumor specimen within any field.

Sections of tracheobronchial tree were examined with a light microscope under 20X and 200X magnification for evidence of pulmonary colonization of osteosarcoma (hematogenous metastasis). Tumor-bearing mice were examined for evidence of local and systemic disease (ie, ulceration, bruising, dependent swelling, lameness, respiratory difficulty, and death).

Statistical analysis—Statistical calculations were performed with a computer software program. Tumor growth delay for groups was computed by means of Kaplan-Meier log-rank survival analysis, followed by the Holm-Sidak method of pairwise multiple comparisons. For the Kaplan-Meier analysis, the interval during which tumor-bearing limbs grew from 8 to 13 mm was used to represent survival (failure). Because values for percentage tumor necrosis were not evenly distributed, percentage tumor necrosis in each group was compared with a Kruskal-Wallis 1-way ANOVA on ranks and results were summarized as median ± SEM. All other values are reported as mean ± SD. Values of P < 0.05 were considered significant for all analyses.

Results

Excluding primary tumors, no gross pathologic change was detected in any mouse. Macroscopic tumors developed in all mice. Hind limbs in which HMPOS cells were injected reached a diameter of 8 mm within a mean ± SD interval of 7.5 ± 0.9 days. Tumors were allowed to develop until the maximum diameter of the injected area of the left hind limb grew to 13 mm (corresponding to a tumor weight of approx 1.0 g), and this size was achieved in all mice.

Intervals for tumor-bearing hind limbs to increase from 8 to 13 mm in diameter were significantly (P < 0.001) longer for mice that received 15-Gy (21.0 ± 5.1 days) and 10-Gy (12.1 ± 3.7 days) radiation therapy, compared with the value in control mice (6.2 ± 2.1 days). The interval to reach 13 mm was also significantly (P < 0.001) longer for mice that received 15-Gy radiation therapy, compared with the value for mice that received only 10 Gy (Figure 1).

All tumors in control mice were graded as highly vascular (ie, subjective score of 4). The mean ± SD number of immunohistochemically identified microvessels in those tumors was 150.3 ± 31.2/200X field (Figure 2). All tumors were characterized as having infiltrative growth patterns. Tumor cells were round or polygonal, with a moderate amount of cytoplasm and round-oval, chromatin-stippled nuclei (Figure 3). Each tumor contained uncalcified bone matrix and bone. No tumor cells appeared to invade the tumor vasculature, which would have indicated hematogenous metastasis. Evidence of macroscopic or microscopic pulmonary metastasis of tumor cells was not detected in any mouse.

Some degree of necrosis was evident in tumors from all treatment groups. Percentage of tumor necrosis was estimated.
not significantly \((P = 0.12)\) different among the groups (median ± SEM values: control mice, 8.4 ± 3.0%; mice that received 10-Gy radiation, 7.9 ± 3.3%; and mice that received 15-Gy radiation, 5.6 ± 4.6). Necrosis was generally evident in linear, focal, or multifocal areas of tumors, and there was no consistent pattern of necrosis in any tumors of any treatment group (Figure 4).

Tumor-related disease was apparent in all mice. All mice developed some degree of lameness as the diameter of the tumor-bearing limbs approached 13 mm. Swelling of the paw (ie, distal to the xenograft) was evident in 1 mouse. This swelling resolved within 24 hours after injection of the HPOMS cells without treatment. Skin changes, respiratory difficulty, bruising, or death attributable to the xenografts or radiation therapy was not detected in any mouse.

Discussion

In the study reported here, tumor size, shape, location, and interval to reach 0.2 g (tumor-bearing limb diameter of 8 mm) were consistent in all mice in which tumors were induced via injection of HPOMS cells into gastrocnemius muscle. The 0.2-g tumors developed within approximately 7 days after injection of cells, which was substantially shorter than intervals reported for canine osteosarcoma xenografts created via SC administration of cells, a process by which comparably sized tumors often develop after 6 to 12 weeks.\(^3\)\(^-\)\(^11\)

The accelerated tumor growth evident in the mice in the present study may have been attributable to the IM injection of our xenografts because rich blood supply within muscles provides stable xenograft reception.\(^19\)

Another explanation could be differences among studies in the concentration or number of osteosarcoma cells injected. We would expect xenografts to develop larger and faster with increasing concentration and volume of the injectant. In fact, although our xenografts developed faster than those of other studies,\(^2\)\(^-\)\(^11\) we used fewer cells per injection \((5 \times 10^5 \text{ cells})\) than were used elsewhere \((10 \times 10^5 \text{ cells to } 100 \times 10^5 \text{ cells})\). The end point of our study was a tumor-bearing limb diameter of 13 mm, representing a tumor that weighed approximately 1.0 g, which is smaller than the end point used in another study.\(^2\) Benefits of rapid growth of tumors and a relatively small tumor size for an end point include shortened investigation time and, consequently, reduced financial costs.

Advantages of our IM xenograft model of canine osteosarcoma in mice included repeatability and uniformity of tumors (rate, location, and shape of development); ease of tumor manipulation, measurement, and treatment; minimal illness in recipient mice; and ease of tumor dissection for postmortem evaluation. Reports\(^2\)\(^-\)\(^11\) of SC canine osteosarcoma xenografts in the flank, interscapular, and proximal femoral regions of mice describe irregular ellipsoid growth, necessitating 3-dimensional measurement of tumors. Although those tumor models effectively produced tumors, substantial
intertumor variation in the shape, growth rate, and location of tumors resulted. The developers of those models measured the tumors with Vernier calipers, and tumor volumes were estimated via mathematical formulations. Measurement with a metric-circles template accurately estimates tumor volume and weight by means of a formula and graph. The metric-circles template approach is much simpler and faster than other techniques that use 3-dimensional measurements made with Vernier calipers. However, the model reported here is limited by tumor size as an end point. Consequently, tumors exceeding 1.0 g made ambulation cumbersome for recipient mice and impaired quality of life such that euthanasia was necessary early in the growth of tumors.

Morbidity and death associated with tumor xenografts have not been well described. Previously our group of researchers reported that 10% of mice (including mice in treatment and control groups) died or required euthanasia before the end of a study because of the degree to which the mice had physically deteriorated. During that study, many mice developed ulceration of the skin surrounding the injection site, and several mice with tumors in the flank suffered tumor invasion into the abdominal cavity and associated fatal intra-abdominal hemorrhage. Ulceration presumably developed because of osmotic necrosis ascribed to a highly concentrated injectant that desiccated surrounding cells, avascular necrosis caused by the xenograft outgrowing its blood supply, or direct invasion of tumor through the skin by seeding of tumor cells in the injection site. No xenograft-associated skin ulceration was detected in the present study. The lack of ulceration may have been attributable to the vascularity of the muscle, the depth of the IM injection (compared with SC injection), or the use of a low concentration of injectant and, therefore, a low risk of injection-site seeding. Alternatively, because the end point of our study involved earlier euthanasia of mice and smaller tumor size than were used in other studies, our IM xenografts may not have had sufficient time to produce ulcerations.

Lameness, which developed in all mice in our study, was the only noticeable impairment. This lameness was believed to be caused by mechanical impingement of the caudal tumor-bearing gastrocnemius muscle on the caudal aspect of the thigh, inhibiting flexion of the stifle joint. Tumor-bearing limbs did not appear to cause pain for the mice, and lameness was only evident when the limb diameter approached 13 mm.

Various models of cancers other than osteosarcoma in the gastrocnemius muscle of mice have been reported and are commonly used for radiation therapy studies. These tumor models are similar in characteristics to the model reported here, and on the basis of our experience, the most aggressive tumors develop to useful size (0.2 g) in only 7 days, resulting in minimal tumor-related disease. Recently, researchers at the University of Florida Comparative Oncology Laboratory investigated the in vitro radiosensitivity of canine osteosarcoma cells. Through this research, calculation of $\alpha$-$\beta$ ratios provided insight into the radioresistance associated with fractionated radiation therapy. Our investigation sought to evaluate the effect of radiation therapy on HMPOS cells in vivo. External-beam radiation therapy results in time- or dose-related effects to surrounding tissues. Therefore, all efforts should be made to exclude healthy tissues from the radiation beam. The IM tumor model reported here had a substantial advantage over other axial xenograft models in that for radiation therapy, the tumor-bearing limb may be isolated from the rest of the mouse, thus minimizing exposure of the remainder of the mouse to radiation and mitigating the development of systemic radiation-associated adverse effects.

Results of our study indicated that 1 radiation dose of 10 or 15 Gy significantly increased intervals for tumor-bearing limbs to increase in diameter from 8 to 13 mm, compared with the interval for mice that did not receive radiation therapy. Furthermore, results of the Kaplan-Meier survival analysis suggested that the delayed growth of tumors in limbs exposed to 10- and 15-Gy radiation was dose dependent.

One limitation of IM xenograft tumor models at any anatomic site is that it is unknown whether the models represent the true architecture of patient bone tumors. Immortalized and transformed cell lines used as substrates in xenograft models can deviate significantly from the native, complex tumor environment, thus making evaluation of the model and any therapeutic response difficult. The primary tumors induced in our study were highly vascular and histologically similar to the original tumors from which the cell lines were developed. Xenografts in our study had osteoblastic differentiation and produced bone and uncemented bone matrix, indicating histologic similarity to naturally developing canine osteosarcoma. Heterotopic tumor models such as that described in our report are inferior to orthotopic (intraosseous) xenografts with respect to approximating spontaneously developing appendicular osteosarcoma.

Mean percentage of tumor necrosis is reportedly approximately 23% for spontaneously developing, untreated canine osteosarcoma. That percentage of necrosis increases to approximately 80% when dogs with spontaneously developing osteosarcoma are treated with external-beam radiation therapy, and the dose required to achieve 80% tumor necrosis is approximately 40 Gy when administered alone. Interestingly, in our study, tumors in control or treated mice did not achieve 25% necrosis. The lower percentage of necrosis detected in our study may have simply been the result of the small size of tumors (which may not have outgrown their blood supply as would have larger, spontaneous tumors in dogs) or differences in radiation dosing (10 or 15 Gy in our study vs 36 to 52 Gy in the other study). Other reports of SC canine osteosarcoma xenografts and IM nonosteosarcoma xenografts indicate a pattern of central tumor necrosis similar to that reported here, although no quantitative measurements were made in those other studies. There was no significant difference in percentage tumor necrosis among groups in the present study. However, because a post hoc power analysis ($\alpha = 0.05$) of these comparisons indicated power was low (0.276), our results must be interpreted with caution. This low power was likely attributable to high variation among mice.
The most devastating aspect of cancer is the emergence of metastasis in organs distant from the primary tumor.1,11 Osteosarcoma is one of the most malignant tumors in humans and other animals and often results in early-stage metastasis to the lungs.1,11 Tumors that grow larger than 2 mm in diameter can already synthesize and secrete angiogenic factors that facilitate invasation, shedding of tumor cells, and the formation of blood vessels (metastases).28 Pulmonary metastasis has developed in athymic mice in which HMPOS cells were used to induce tumors, with micrometastases evident at 4 weeks and macrometastases at 6 weeks after inoculation.2 In the present study, pulmonary metastasis was not detected in any mouse. The lack of metastasis was likely a result of the lower number of tumor cells injected (5 × 10⁵ cells in our study vs 50 × 10⁵ cells in other studies18,19) and a short survival time. In control mice, the end point occurred approximately 2 weeks after injection of HMPOS cells, and although some treated mice did survive for 4 weeks, this survival was dependent on local tumor control that likely impacted tumor progression to the metastatic stage.

Excluding primary tumors, no gross pathologic change was detected during necropsy of any mouse in our study. We did not perform histologic assessment of any organ other than the lungs. However, given that another investigation in which the SC HMPO xenograft model was used did not detect metastasis to any other organs when pulmonary metastasis was evident, it is unlikely that metastasis to other organs would have occurred in our study without pulmonary metastasis being evident.

References


