Intrinsic radiosensitivity and repair of sublethal radiation-induced damage in canine osteosarcoma cell lines

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Objective—To characterize the radiosensitivity and capacity for sublethal damage repair (SLDR) of radiation-induced injury in 4 canine osteosarcoma cell lines.

Sample Population—4 canine osteosarcoma cell lines (HMPOS, POS, COS 31, and D17).

Procedures—A clonogenic colony-forming assay was used to evaluate the cell lines’ intrinsic radiosensitivities and SLDR capacities. Dose-response curves for the cell lines were generated by fitting the surviving fractions after radiation doses of 0 (control cells), 1, 2, 3, 6, and 9 Gy to a linear-quadratic model. To evaluate SLDR, cell lines were exposed to 2 doses of 3 Gy (split-dose experiments) at an interval of 0 (single 6-Gy dose), 2, 4, 6, or 24 hours, after which the surviving fractions were assessed.

Results—Mean surviving fraction did not differ significantly among the 4 cell lines at the radiation doses tested. Mean surviving fraction at 2 Gy was high (0.62), and the α/β ratios (predictor of tissue sensitivity to radiation therapy) for the cell lines were low (mean ratio, 3.47). The split-dose experiments revealed a 2.8- to 3.9-fold increase in cell survival when the radiation doses were applied at an interval of 24 hours, compared with cell survival after radiation doses were applied consecutively (0-hour interval).

Conclusions and Clinical Relevance—Results indicated that these canine osteosarcoma cell lines are fairly radioresistant; α/β ratios were similar to those of nonneoplastic, late-responding tissues. Future clinical investigations should involve increasing the fraction size in a manner that maximizes tumor killing without adverse effects on the nonneoplastic surrounding tissues. (Am J Vet Res 2008;69:1197–1202)
Materials and Methods

Canine osteosarcoma cell lines—Four canine osteosarcoma cell lines (POS,\textsuperscript{a} HMPOS,\textsuperscript{b} COS 31,\textsuperscript{b} and D17\textsuperscript{c}) were used for the study. The POS cell line was originally developed from a primary osteosarcoma of the proximal portion of a femur of a 1.5-year-old mongrel dog.\textsuperscript{24} The HMPOS (highly metastasizing POS) cell line was a pulmonary metastatic subtype of the POS cell line.\textsuperscript{25} The COS 31 cell line was derived from a naturally occurring osteosarcoma in a dog.\textsuperscript{26} The D17 cell line was established from a canine pulmonary metastatic tumor.

The POS and HMPOS cell lines were maintained on growth RPMI 1640 medium that contained (on a per-liter basis) 100 mL of 10% heat-inactivated FBS, 20 mL of vitamin solution (100X), 10 mL of l-glutamine (200mM), 10 mL of sodium pyruvate (100mM), 10 mL of nonessential amino acid solution (10mM), and 10 mL of penicillin-streptomycin (10,000 µg/mL).\textsuperscript{27} Doubling times for POS and HMPOS cells were 38.6 and 39.3 hours, respectively. These POS and HMPOS cell lines were chosen to evaluate whether a metastatic cell subtype (HMPOS) differed from that of its parent cell line (POS). The COS 31 cell line was maintained on Dulbecco modified Eagle medium with 10% FBS (100 mL of 10% non–heat-inactivated FBS/L of medium).\textsuperscript{28} Doubling time for COS 31 cells was 38.6 hours. The D17 cell line was maintained on Dulbecco modified Eagle medium with 10% FBS (100 mL of 10% non–heat-inactivated FBS/L of medium).\textsuperscript{29} Doubling time for D17 cells was 25.2 days. All incubations were performed with standard conditions of 37°C and 5% CO\textsubscript{2}.

Radiation dose-response assay—For each cell line, 3 independent stock flasks were developed and maintained, each originating from a different cell passage. Stock cultures were rinsed with Hank’s balanced salt solution\textsuperscript{30} and were treated with 0.25% trypsin.\textsuperscript{31} The cell concentration for each stock culture was established via cell counting on a hemacytometer, and serial dilutions were performed until a working concentration of approximately 100 cells/mL was achieved. Preliminary data were used to determine optimal seeding numbers for the control and irradiated groups for each cell line. For each of the 3 stock flasks maintained of each cell line, ten 60-mm Petri dishes\textsuperscript{32} were seeded at 350 cells/plate and incubated overnight for approximately 18 hours.

The plates were then irradiated at ambient temperature and pressure by use of a 6-MV linear accelerator\textsuperscript{33} at the University of Florida McKnight Brain Institute research facility. The radiation dose was given at a rate of 400 cGy/min. Radiation from a 6-MV linear accelerator does not reach maximum dose until it has traversed 1.5 cm of tissue-equivalent thickness. To ensure that the proper dose was delivered, a 1.5-cm water-equivalent lucite plate was placed on top of the Petri dishes and a 5-cm water-dense plate was placed underneath the Petri dishes to ensure full backscatter. Radiation doses used were 0, 1, 2, 3, 6, or 9 Gy. Control cells (radiation dose, 0 Gy) were transported along with the other treated cells but remained outside the radiation vault during treatments. All cells were returned to the incubator, and the medium was completely changed weekly or biweekly, as needed. The plates remained in the incubator until the colonies had grown to a size that was sufficient for counting without convergence (approx 10 to 14 days after initial seeding). At the appropriate time of counting, each plate was rinsed twice with PBS solution, fixed with 70% ethanol, and stained with 0.1% crystal violet. The Petri dishes were examined at low magnification (10X), and colonies composed of 50 or more cells were scored as surviving colonies.

For each of the 3 stock flasks in a given cell line, 2 Petri dishes were designated for each radiation treatment dose. The experiment was performed 3 times, yielding 18 samples for each cell line at each dose.

Assessment of SLDR ability (split-dose repair assay)—For the SLDR experiment, plates were seeded in the same manner as for the radiation dose-response experiment. Approximately 18 hours after seeding, all plates except the control plates were irradiated with a 3-Gy radiation dose in the same manner as for the dose-response experiment; plates were then returned to the incubator. A second 3-Gy dose was delivered after cells were incubated for 0, 0.5, 1, or 2 Gy in a single procedure, or 2, 4, 6, or 24 hours. The plates were then incubated for 10 to 20 days, and colonies were counted by use of the same protocol as described above.
for the radiation dose-response experiment. For each of the 3 stock flasks in a given cell line, 2 plates were designated to receive the second treatment at each time point. The experiment was performed 3 times, yielding 18 samples for each cell line at each time point.

For both experiments, the plating efficiency (ie, PE) was calculated\(^\text{11,24}\) for each of the control plates:

\[
\text{PE} = \frac{\text{(number of surviving colonies)}}{\text{(number of cells seeded onto that plate)}}
\]

The SF for each of the radiation-dosed plates associated with each cell line was then calculated\(^\text{11,24}\):

\[
\text{SF} = \frac{\text{(PE of treated sample)}}{\text{(PE of control sample)}}
\]

Data analysis—Statistical analysis was performed by use of computer software.\(^\text{6b}\) A repeated-measures ANOVA was used to analyze the response to dose escalation (with dose as the repeated measure) and the split-dose experiment data (with time in hours as the repeated measure). To estimate the \(\alpha/\beta\) ratios among groups for the dose-response experiment, the median SFs for each cell line were regressed against dose by use of a standard linear quadratic model (SF = \(e^{(\alpha \cdot \text{dose} - \beta \cdot \text{dose}^2)}\)).

A value of \(P < 0.05\) was interpreted as significant.

Results

For the dose-response experiment, the repeated-measures ANOVA revealed that SFs significantly \((P < 0.001)\) decreased as dose increased. The mean SF did not differ significantly \((P = 0.981)\) among the 4 cell lines at any of the radiation doses tested. Dose response curves and corresponding SEM values were evaluated (Figure 1; Table 1). The shape of the downward curve did not differ \((P = 0.111)\) among the 4 cell lines. Values of SF, \(\alpha\), \(\beta\), and \(\alpha/\beta\) ratios for each of the cell lines were calculated (Table 2).

For the split-dose experiment, repeated-measures ANOVA revealed a significant \((P < 0.001)\) increase in SF for all 4 cell lines with increasing intervals between radiation doses (Figure 2). The shape of the upward curve differed \((P = 0.003)\) among the 4 cell lines. In all 3 split-dose experiments, there was a sharp increase in the SF of the COS 31 cell line in the plates that received the second dose of radiation after an interval of 24 hours, compared with the SF of those cells in the plates that received the second dose of radiation after an interval of 6 hours. Compared with the findings in plates that received the 6-Gy dose of radiation without interval (0-hour interval groups), the fold increase in SF resulting from the 24-hour delay before application of the second 3-Gy dose was 2.79, 2.92, 3.34, and 3.94 for the HMPOS, POS, D17, and COS 31 cell lines, respectively.

Table 1—Standard error of the mean values for SF for each of 4 canine osteosarcoma cell lines after radiation treatments of 1, 2, 3, 6, or 9 Gy in a dose-escalation experiment.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>Radiation dose (Gy)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>POS</td>
<td>0.04</td>
</tr>
<tr>
<td>HMPOS</td>
<td>0.03</td>
</tr>
<tr>
<td>COS 31</td>
<td>0.01</td>
</tr>
<tr>
<td>D17</td>
<td>0.04</td>
</tr>
</tbody>
</table>

Table 2—Mean ± SEM SF values, \(\alpha\) and \(\beta\) values, and the \(\alpha/\beta\) ratio for each of 4 canine osteosarcoma cell lines after radiation treatment of 2 Gy. Values were derived from the linear-quadratic equation fitted to the radiation dose-response curve. The mean \(\alpha/\beta\) ratio for the 4 cell lines was 3.47.

<table>
<thead>
<tr>
<th>Cell line</th>
<th>SF</th>
<th>(\alpha)</th>
<th>(\beta)</th>
<th>(\alpha/\beta) ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>POS</td>
<td>0.60 ± 0.05</td>
<td>0.16</td>
<td>0.05</td>
<td>3.5</td>
</tr>
<tr>
<td>HMPOS</td>
<td>0.61 ± 0.04</td>
<td>0.16</td>
<td>0.04</td>
<td>3.8</td>
</tr>
<tr>
<td>COS 31</td>
<td>0.64 ± 0.06</td>
<td>0.18</td>
<td>0.03</td>
<td>5.6</td>
</tr>
<tr>
<td>D17</td>
<td>0.63 ± 0.03</td>
<td>0.08</td>
<td>0.08</td>
<td>1.0</td>
</tr>
</tbody>
</table>

Figure 1—Dose-response curve (SF vs radiation dose) depicting the radiosensitivity of 4 canine osteosarcoma cell lines derived from data collected after radiation treatments of 1, 2, 3, 6, or 9 Gy in a dose-response experiment. Control cells were not irradiated (dose, 0 Gy). For each of 3 stock flasks of a given cell line, 2 Petri dishes were designated for each radiation treatment dose. The experiment was performed 3 times, yielding 18 samples for each cell line at each dose.

Figure 2—Mean ± SEM SF for each of 4 canine osteosarcoma cell lines derived from data collected by use of a split-dose repair assay. All groups were treated with an initial radiation dose of 3 Gy followed by a second dose of 3 Gy after an interval of 0 (ie, 6-Gy dose administered during 1 treatment), 2, 4, 6, or 24 hours. For each of 3 stock flasks of a given cell line, 2 plates were designated to receive the second treatment at each time point. The experiment was performed 3 times, yielding 18 samples for each cell line at each time point.
Discussion

For the 4 canine osteosarcoma cell lines investigated in the present study, establishment of their SF$_2$ values provides valuable information regarding the intrinsic radiosensitivities of those cells. From radiosensitivity data for a wide variety of human tumors, Fertil and Malaise$^{16}$ determined that the SF$_2$ value is useful as an indicator of tumor cell radiosensitivity and of clinical response to radiation therapy. In that study, SF$_2$ ranged from 0.13 to 0.90 for various cell lines and cell lines with lower SF$_2$ values were clinically more radiosensitive.$^{16}$ In the present study, the SF$_2$ values for the 4 canine osteosarcoma cell lines ranged from 0.60 to 0.64, which is a somewhat narrow range, and the mean value was 0.62. On the basis of the findings of the study by Fertil and Malaise,$^{16}$ the values for the canine osteosarcoma cell lines indicated that they are moderately radioresistant. The mean SF$_2$ for the canine osteosarcoma cells (0.62) was higher than that reported for human osteosarcoma cells (0.47$^{23}$ and 0.37$^{20}$); thus, the data from the present study suggest that canine osteosarcoma cells may be more radioresistant than human osteosarcoma cells. The relative radioresistance of canine osteosarcoma cell lines in our study is clinically supported by the fact that, in general, dogs with osteosarcoma do not respond well to curative-intent fractionated radiation therapy.$^{18,27}$ Interestingly, the SF$_2$ values for the cell lines that originated from primary tumors (POS, 0.60; COS 31, 0.63) were similar to values from cells that originated from metastatic tumors (HMPOS, 0.61; D17, 0.64). Although the information regarding the metastatic cells is less clinically relevant, our findings suggest that the selection for metastatic cell types within a primary tumor may not affect cellular radiosensitivity.

In the linear-quadratic curves generated in the present study, the $\alpha$ value represented irreversible cellular injury, whereas the $\beta$ value represented sublethal (repairable) cellular injury.$^{17}$ The $\alpha/\beta$ ratio can be used as a predictor of tissue sensitivity to radiation therapy, specifically a predictor of the effect of a fractionation schedule on cell survival.$^{13}$ For instance, tumors with a high $\alpha/\beta$ ratio (mimicking early-responding tissues) are less sensitive to dose-per-fraction changes. In this instance, hyperfractionated radiation therapy, which incorporates smaller-than-standard doses per fraction, may be used to preserve surrounding nonneoplastic tissues without compromising the tumor cell kill effect.$^{15}$ Conversely, tumors with a low $\alpha/\beta$ ratio (mimicking late-responding tissues) are sensitive to changes in a fractionation schedule. Hypofractionated radiation therapy, which incorporates larger-than-standard doses per fraction, can be used in these situations, provided that the nonneoplastic tissue tolerance is not exceeded.$^{15}$ Typically, normal nonneoplastic late-responding tissues, such as nervous tissues, have a low $\alpha/\beta$ ratio (eg, $<5$).$^{13}$ The mean $\alpha/\beta$ ratio for canine osteosarcoma cells in the present study was also low (3.47). When evaluated concurrently, the low $\alpha/\beta$ ratio and high SF$_2$ of those tumor cells indicated that canine osteosarcomas are difficult to treat effectively with conventional fractionated radiotherapy protocols (eg, 3.0 Gy $\times$ 19 fractions in dogs$^{27}$) and that larger-than-standard doses per fraction are needed to induce greater tumor cell killing. These findings suggested that conformal radiation procedures that are capable of delivering markedly large fractions while sparing normal surrounding tissues (eg, stereotactic radiosurgery$^{19}$) may be more effective in achieving tumor control.

In the present study, there was no significant difference in radiosensitivity among the 4 canine osteosarcoma cell lines. Potential clinical differences in the inherent radiosensitivities of naturally occurring osteosarcomas have been suggested. In a recent investigation,$^{27}$ a variable response to radiation was detected in clinically affected dogs. In that retrospective study,$^{27}$ the effectiveness of curative-intent radiotherapy in 14 dogs with osteosarcoma was evaluated; some primary tumors (approx a third of those monitored) were controlled via radiotherapy, and those affected dogs died of metastatic disease or other causes without any evidence of local tumor recurrence. However, it was also reported that tumors in approximately two thirds of the dogs appeared to be more resistant to the effects of radiation, and local tumor progression was evident in the dogs prior to their deaths. Ideally, comparison of a large number of canine osteosarcoma cell lines should be performed before concluding that inherent differences in radiosensitivity among them do not exist.

To evaluate the ability of the cell lines to repair the damage induced by radiation, split-dose experiments were performed in the present study. The experiments involved exposing the cell lines to an initial dose of radiation, allowing a variable amount of time to elapse to allow for SLDR, and irradiating the cell lines with a second dose of radiation.$^{17}$ Repair of sublethal radiation-induced damage accounted for the initial detectable increase in SF (ie, $<4$ hours) in the present study. The phenomenon of reassortment is a likely explanation for the decrease in SF that occurred between cell lines allowed an interdose interval of 4 hours and those allowed an interdose interval of 6 hours, particularly for the COS and POS cell lines. This reassortment is typically the result of cells that are in a resistant phase (S phase) of the cell cycle becoming redistributed into a more sensitive phase (M phase) with a resultant increase in cell killing. Further increases in SF in cell lines detected with intervals $>12$ hours between radiation doses more likely represented tumor cell repopulation (because repair should be complete within the first 12 hours following irradiation). In our study, when the SF of the control cells (0-hour interval between irradiation treatments [ie, both 3-Gy doses given in 1 treatment]) was compared with SF of the cells that received the second dose of radiation after a 24-hour delay, there was a 2.8- to 3.9-fold increase in colony survival, depending on the cell line. Among the cell lines evaluated, COS 31 had the greatest fold increase (3.9) and HMPOS had the least fold increase (2.8). There are several DNA repair mechanisms that could account for the differences observed. For example, it is well established that radiation-induced DNA damage initiates a signal transduction pathway that increases the expression of the tumor suppressor gene p53.$^{28}$ Accumulation of p53 protein leads to arrest of the G1 phase of the cell cycle that may facilitate DNA repair.$^{29,30}$ Results of some studies$^{31,32}$
suggest that mutations in the p53 gene are associated with increased radioresistance to killing. Although it was beyond the scope of the present study, investigation into the DNA repair mechanisms of the 4 evaluated osteosarcoma cell lines is warranted.

Theoretically, 95% of cell repair will be complete within 12 hours after a radiation insult in human tissues. Therefore, the maximum amount of recovery for the cell lines examined in our study should have been achieved by application of a 24-hour dosing interval. In a study to evaluate the effect of curative-intent radiation therapy in dogs with osteosarcoma, administrations of 2.7- to 3.0-Gy fractions were separated by an interval of 24 hours, typically on a Monday-through-Friday schedule. The use of hyperfractionation (eg, twice-daily fractions) in the treatment of osteosarcoma in humans has been reported. In another investigation in humans with osteosarcoma of the extremities, tumors were irradiated to a median dose of 60 Gy (range, 40 to 68 Gy) with either daily fractions of 2.5 to 3.0 Gy (5 d/week) or 1.25 to 1.5 Gy delivered twice daily at an interval of at least 4 hours (10 fractions/week). No correlation between the rate of local treatment failure and regimen of fractionation was identified. Hyperfractionation protocols for osteosarcomas in dogs have not been reported, to our knowledge; however, based on the low α/β ratios determined for the 4 canine osteosarcoma cell lines in our study, we speculate that hyperfractionation would not offer substantial benefit.

There are several limitations to the present study. First, in vitro cell culture experiments do not reflect the microenvironment present in naturally occurring tumors, and tumor cells irradiated in vitro have decreased SLDR capacity, compared with that of cells irradiated in vivo, possibly because cells in vitro lack 3-dimensional cellular contact. Naturally occurring tumors also have areas of tissue hypoxia that make tumor cells less susceptible to the effects of radiation. Second, it would have been ideal in the SLDR experiment in the present study to have included treatment groups that were exposed to the second 3-Gy dose after intervals >6 hours and <24 hours (eg, at 12 hours after the first dose because repair is typically complete by 12 hours after irradiation); however, we did not have access to the linear accelerator at the 12-hour time point. The findings of the present study are consistent with clinical observations that appendicular osteosarcoma in dogs is a moderately radioresistant tumor, and these data may explain why conventional fractionated protocols have not been uniformly successful in treatment of affected dogs. Future clinical efforts should involve increasing the fraction size in a manner that spares the surrounding nonneoplastic tissues from radiation-induced damage.

References