The Frequency of Micronuclei in Lymphocytes of Dogs with Osteosarcoma: A Predictive Variable for Tumor Response during Cisplatin Chemotherapy

Kevin A. Hahn, Alfred M. Legendre, and James R. Talbott

Abstract

To our knowledge, there are no features identifiable at the time of diagnosis or during treatment that can assist the clinician in predicting the response to cisplatin therapy in dogs with osteosarcoma. In this study, we describe a direct relationship between the percentage of G0 lymphocytes containing micronuclei following exposure to one dose of cisplatin in vivo and tumor response in dogs with osteosarcoma. The response of tumors to chemotherapy is thought to be a function of the drug's pharmacological properties (e.g., peak plasma concentration and elimination half-life); however, a relationship between platinum DNA adduct levels in leukocyte DNA and tumor response has been observed by others, suggesting that clinical resistance to platinum drugs is attributable to DNA repair functions of the host, and thus the degree of cytotoxicity is similar across all cell types. Our results support this hypothesis. Those dogs receiving cisplatin chemotherapy and having a micronuclear frequency of greater than 10% had median remission and survival times of 68.3 and 79.0 weeks, respectively, whereas those dogs with a micronuclear frequency of less than 10% had median remission and survival times of 14.1 and 17.9 weeks, respectively.

Introduction

Micronuclei are small, intracytoplasmic bodies containing DNA that occur in interphase cells when acentric chromosome fragments or whole chromosomes are not incorporated into the daughter nuclei formed at mitosis (1). Therefore, micronuclei can be used as a relatively simple means of detecting and quantifying chromosome aberrations in cells following exposure to cytotoxic treatment (2, 3).

To our knowledge, there are no features identifiable at the time of diagnosis or during treatment that can assist the clinician in predicting the response to cisplatin (Platinol, cis-diaminedichloroplatinum II; Bristol-Myers Squibb Oncology Division, Evansville, IN) therapy in dogs with osteosarcoma. We have previously demonstrated that there is a direct relationship between the percentage of micronuclei formed in normal G0 canine lymphocytes and increasing doses of cisplatin in vitro (4). In this study, we describe a direct relationship between the percentage of G0 lymphocytes containing micronuclei following exposure to one dose of cisplatin in vivo and tumor response in dogs with osteosarcoma.

Materials and Methods

Patient Population. Thirteen dogs with previously untreated, histologically confirmed appendicular osteosarcoma were evaluated at the University of Tennessee Veterinary Teaching Hospital. All dogs were staged by survey thoracic radiography (three views) and abdominal radiography (two views), bone radiographs of the primary tumor site, and bone scintigraphy (5). A complete history, physical examination, complete blood count, serum biochemistry profile, electrocardiogram, and urinalysis were done on each dog.

Treatment Protocol. Dogs without detectable metastatic disease or synchronous primary tumors had the affected limbs amputated. The following day, the dogs were given 60 mg/m2 cisplatin every 3–4 weeks to a cumulative dose of 240 mg/m2 (6). All dogs were restaged (e.g., physical examination and survey thoracic radiography) 4, 12, 24, 36, 48, 72, and 96 weeks following amputation and initiation of chemotherapy. Dogs that died or were euthanized had complete necropsies. Remission time was defined as the time from amputation to detection of metastasis(es) by physical examination or radiographs. Survival time was defined as the time from amputation to death.

Micronucleus Assay. The cytokinesis block micronucleus assay was used in this study to quantify the frequency of micronuclei formed in postmitotic cells (7–9). Briefly, 2 ml blood were taken before each dose of cisplatin and again at 4, 12, 24, and 36 weeks following the fourth dose of cisplatin chemotherapy (i.e., samples were obtained at 0, 4, 8, 12, 16, 24, 36, and 48 weeks following amputation). Lymphocytes were separated by density centrifugation and suspended in RPMI 1640 (Life Technologies, Inc., Gaithersburg, MD). Lymphocyte cultures were supplemented with l-glutamine, HEPES buffer, 10% FCS, antibiotics, heparin, and mitogen. Prepared cultures were set in triplicate and incubated at 37°C in a 5% CO2 humidified atmosphere. Cytochalasin B (Sigma Chemical Co., St. Louis, MO) was added 24 h before harvest to each culture flask at a concentration of 6 μg/ml to block cytokinesis (2–4). The stock...
solution of cytochalasin B was prepared by dissolving 1.0 mg of the lyophilized material in 1.1 ml DMSO and kept at \(-70^\circ \text{C}\). An aliquot of the stock solution was diluted in fresh RPMI 1640 prior to addition to cell cultures. At the appropriate time, cells were harvested, the nuclei were fixed twice in alcohol (3 methanol:1 acetic acid) using standard cytogenetic procedures, and slides were prepared and stained with 4% Giemsa (2-4).

The total number of binucleate cells with micronuclei and the number of micronuclei per binucleate cell were determined from coded slides using 2000 randomly selected binucleate cells scored per culture from four slides. The criteria for identifying binucleate cells were as follows: (a) approximately the same diameter for both nuclei; (b) nuclei had to be perfectly separated from one another, or at least the nuclear membrane in both had to be perfectly visible; and (c) no filaments or bridges binding the two nuclei (10). The criteria for identifying multinucleated cells were the presence of three or more nuclear structures in the same cell. These structures could be nuclei (approximately the same size). When micronuclei were present, at least two other nuclei had to be present in the same cell (10).

**Statistical Analysis.** Differences in the micronuclear frequency between the various time intervals were tested with nonparametric methods (Kruskal-Wallis, Mann-Whitney, and Wilcoxon statistics) and (repeated measures) ANOVA (11). Because the distribution of variances between the micronuclear frequencies was skewed (Bartlett’s test for homogeneity of variances, \(P < 0.0005\)), all analyses were applied to log-transformed observations (11). To determine whether a relationship existed between remission and survival times with micronuclear frequencies obtained at each time interval, scatter plots were generated. The Kaplan-Meier method was used to generate outcome distribution curves to illustrate any relationship(s) between remission or survival times with a particular micronucleus frequency. For all analyses, significance was established at \(P < 0.05\).

### Results

The 13 dogs had a mean age of 7.8 (range, 4.5-11.4) years and a mean body weight of 35.7 (range, 22.0-54.6) kg. Nine dogs completed the scheduled four doses of cisplatin; 4 dogs did not complete the scheduled protocol, because they developed metastasis. In total, 43 doses of cisplatin were given (mean, 3.3 doses per dog; range, 1-4 doses).

The micronuclear frequencies obtained from each dog at each evaluation period are summarized in Table 1. The mean micronuclear frequency of G0 lymphocytes before the first dose of cisplatin was 0.07 ± 0.05 (\(n = 13\)) and differed significantly from the mean micronuclear frequencies obtained after all cisplatin treatments (Table 1). There were no significant differences between any of the mean micronuclear frequencies at any time following the first cisplatin chemotherapy. All dogs had an increase in micronuclear frequency after the first dose of cisplatin. The increase was sustained throughout and following the treatment period (Fig. 1). Those dogs with micronuclear frequencies of greater than 10% after the first dose of cisplatin had significantly longer remission and survival times than those dogs with micronuclear frequencies of less than 10% (Fig. 1). Multiple regression analysis using a generalized linear model evaluating the change in micronuclear frequency from the beginning of therapy to the completion of therapy showed no significant relationship to age, sex, or cumulative dose. When remission and survival times were stratified by a micronuclear frequency of ≥10% (Fig. 2), a significant difference between the two groups was observed (\(P < 0.05\)). Those dogs receiving cisplatin chemotherapy and having micronuclear frequencies of greater than 10% had median remission and survival times of 68.3 and 79.0 weeks, respectively, whereas those dogs with micronuclear frequencies of less than 10% had median remission and survival times of 14.1 and 17.9 weeks, respectively (Table 2).
Discussion

Our findings indicate that administration of cisplatin to dogs induces chromosome damage in circulating G0 lymphocytes, as evidenced by the formation of micronuclei on stimulation in vitro. The percentage of micronuclei in G0 lymphocytes after one dose of cisplatin is predictive of tumor remission and survival time.

Osteosarcoma in dogs closely resembles osteosarcoma in humans (12, 13). Osteosarcoma is estimated to occur in more than 6000 dogs in the United States per year, and that figure may be increasing with the growing popularity of large and giant breeds. Dogs with osteosarcoma constitute approximately 1–3% of all canine cancers and 85–95% of all primary bone tumors (14). As with humans, males are affected 2:1 over females. Osteosarcoma in humans is generally a disease of adolescence and is a rare tumor affecting only 2000–2200 people per year in the United States (12, 13).

Osteosarcoma in dogs and humans is virtually indistinguishable clinically and histopathologically (12). Primary sites of development in the dog are the metaphyses of the long bones (distal portions of the radius and ulna, proximal portion of the humerus, and proximal and distal portions of the femur and tibia) and, less frequently, the axial skeleton. Forelimb lesions are more common than hind limb lesions (1.7:1.0). Similar to humans, common survey radiographic abnormalities suggesting osteosarcoma include pronounced cortical bone lysis, aggressive, spiculated periosteal new bone formation, and pathological fractures. A definitive diagnosis of primary, multicentric, metachronous, or metastatic osteosarcoma is made by examination of biopsy specimens (incisional or excisional).

Because microscopic occult metastatic disease is present in 80–90% of patients at presentation (14), some form of systemic therapy is necessary if survival is to be improved. With amputation alone, the 1- and 2-year survival rates are estimated to be 11.5% and 2%, respectively, and the mean survival time is estimated to be 19.2 weeks (14). Cisplatin has shown exceptional efficacy as an adjuvant to surgery in humans and dogs with appendicular osteosarcoma (15, 16). With adju-

### Table 2

<table>
<thead>
<tr>
<th>Micronuclei</th>
<th>Median remission time (wk) (range)</th>
<th>Median survival time (wk) (range)</th>
</tr>
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<tbody>
<tr>
<td>&lt;10% (n = 7)</td>
<td>14.1 (3.7–28.1)</td>
<td>17.9 (4.4–36.6)</td>
</tr>
<tr>
<td>&gt;10% (n = 6)</td>
<td>68.3 (22.1–88.3)</td>
<td>79.0 (22.1–149.7)</td>
</tr>
</tbody>
</table>

P = 0.012; Hazard ratio = 0.27; 95% CI = 0.09–0.72.

*The hazard ratio, sometimes called relative risk, means that, on average, dogs with micronuclear frequencies >10% relapse (e.g., have evident metastatic disease) or die at 0.27 and 0.26 times the rate of dogs with micronuclear frequencies <10%, respectively.
vant cisplatin chemotherapy, 38–62% 1-year survival rates have been reported. Similarly, the overall mean survival time for cisplatin-treated dogs in our study was 46.6 weeks, with a 1-year survival rate of 38.7%.

Cisplatin is an inorganic heavy metal complex with a broad spectrum of antitumor activity (17). Intracellularly, the drug reacts with water; the chloride ligands are displaced, and positively charged aquated species are generated. These reactive intermediates attack nucleophilic sites on the DNA molecule, particularly the N7 positions of guanine and adenine, producing intrastrand and interstrand cross-links as well as DNA-protein cross-links (18). These aberrations can be observed cytogenetically as micronuclei (4). Micronuclei are small, intracytoplasmic bodies containing DNA that occur in interphase cells when acetic chromosome fragments or whole chromosomes are not incorporated into the daughter nuclei formed at mitosis (1). Therefore, micronuclei can be used as a relatively simple means of detecting and quantifying chromosome aberrations in cells following exposure to cytotoxic treatment (2-4).

Clinical resistance to platinum compounds has been attributed to tumor cell resistance to the drug (18). The response of tumors to chemotherapy is thought to be a function of the drug’s pharmacological properties (e.g., peak plasma concentration and elimination half-life; Ref. 18). However, pharmacological data were available in all dogs, and no significant differences were observed in parameters such as peak plasma concentration and area under the curve when dogs were treated with different cisplatin concentrations and area under the curve when dogs were treated with the same cisplatin dose. The level of DNA damage measured in peripheral blood leukocyte DNA and tumor response has been observed by others (20). These observations and our studies support a hypothesis that clinical resistance to platinum drugs is attributable to DNA repair functions of the host, and thus the degree of cytotoxicity is similar across all cell types (20, 21). From our results, the level of DNA damage measured in peripheral blood G0 lymphocytes following the first dose of cisplatin was directly related to tumor response. The fact that this relationship is not influenced by subsequent cisplatin doses (i.e., chromosomal damage induced by cisplatin is not a function of cumulative dose) is the subject of further investigations by our laboratory.

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References

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