Experimental Chemotherapy against Canine Mammary Cancer Xenograft in SCID Mice and Its Prediction of Clinical Effect

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(Received 26 December 2000/Accepted 10 April 2001)

ABSTRACT. The effectiveness of 6 antitumor agents has been evaluated for canine mammary gland tumor (CMG-6) serially transplanted into severe combined immunodeficiency (SCID) mice. CMG-6 diagnosed as a solid carcinoma was subcutaneously transplanted into SCID mice and six antitumor agents were intravenously given to the mice as a single injection. The effectiveness was evaluated by treatment group/Control group percent (T/C %) and statistical significance determined by Mann-Whitney’s U-test in tumor volume. The minimum effective doses (MEDs; mg/kg) of mice were as follows: cyclophosphamide (CPM) 65, doxorubicin (DXR) 6, cisplatin (CDDP) 5, vincristine (VCR) 1.6, vinblastine (VLB) more than 5.5, 5-fluorouracil (5-FU) 105. Clinical effects of the drugs were predicted based on area under the curve (AUC) of dogs given a clinical dose (AUCdog)/AUC of mice given a MED (AUCmouse) ratios from published references. The AUC ratios were as follows; CPM 2.24, DXR 0.19, CDDP 1.20, VCR 0.04, VLB <1.24 and 5-FU 1.15. Drugs indicating more than 1.0 in AUCdog/AUCmouse ratio were CPM, CDDP and 5-FU, and would be suggested as effective in the original patient with CMG-6. The combination chemotherapy using clinically equivalent doses in CDDP and CPM, which were the two highest values in AUCdog/AUCmouse ratio by single agent therapy, was performed and shown to have additional effects as compared to the responsiveness of each agent against CMG-6.

KEY WORDS: canine, chemotherapy, mammary cancer, SCID mouse, xenograft.

Canine mammary gland tumors are very common and have been reported to comprise of about 50% of all neoplasms in the female dog [2]. Approximately half of the mammary gland tumors that occur in the bitch are considered to be malignant [10, 11, 22]. In canine mammary gland tumors, clinical features vary from highly malignant to benign. Therefore, therapy is dependent on their histologic grades or clinical stages. Surgery is the first choice of treatment for canine mammary gland tumors, with the exception of patients with inflammatory carcinomas or distant metastasis [11, 19]. Especially for a highly malignant or an invasive tumor, some adjuvant therapies after surgery, such as radiation therapy, chemotherapy and so on, would be chosen as needed. In some studies, antitumor activities were indicated in the treatment of dogs with mammary adenocarcinoma using doxorubicin, cyclophosphamide or cisplatin as a single agent [19]. However, there is no comparison of the effectiveness of each drug because there has been no controlled clinical trials.

On the other hand, there are many reports with regard to the evaluation of antitumor drugs and that predictability for clinical effects by using a tumor xenograft system. To investigate the usefulness of clinically available agents against canine mammary cancers, a canine mammary gland tumor xenograft (CMG-6) was established by transplantation into SCID mice. In the present study, the chemosensitivity of six antitumor agents against CMG-6 was evaluated, and the clinical effectiveness in the patient was predicted. Also, the additional effectiveness of the combination with chemotherapy against CMG-6 was proven in this study.

MATERIALS AND METHODS

Tumor xenograft: A canine mammary gland tumor established as a xenograft in SCID mice was used. The xenograft designated as CMG-6 (Canine Mammary Gland tumor-6) is histologically diagnosed as a solid carcinoma. The canine patient was an 11-year-old unspayed Shetland Sheepdog weighing 9.5 kg and the specimen of CMG-6 was resected from the left inguinal mammary gland at surgery. Until then, the patient had not had chemotherapy performed. After surgery the patient showed pulmonary metastasis and died at approximately 4 months. This tumor has been maintained by serial subcutaneous transplantation in SCID mice every month.

Mice: Six- to seventeen-week-old female BALB/cA-scid mice (the Central Institute for Experimental Animals, Kawasaki) were used. The mice were maintained in specific pathogen-free conditions: temperature of 24 ± 1°C, humidity of 40–70% and 12 hr-light and dark cycle. Two or three mice were put in one cage (mouse S cage, Clea Japan Inc., Tokyo) and the cages were set on a laminar flow rack. Animals were fed with sterilized food (CE-2, Clea Japan Inc., Tokyo) and water ad libitum.

Antitumor agents: All six drugs used were in a form for
Clinical use. Cyclophosphamide (CPM), vincristine (VCR) and vinblastine (VLB) were purchased from Shionogi & Co., Osaka, doxorubicin (DXR) and 5-fluorouracil (5-FU) from Kyowa Hakko Kogyo Co., Tokyo and cisplatin (DDP) from Nihon Kayaku Co., Tokyo, Japan. All drugs were dissolved in sterile 0.85% NaCl solution just before use, except for CPM, which was dissolved in distilled water for injection.

Chemotherapy: The tumor was cut into 3 × 3 × 3 mm in size and one fragment was subcutaneously transplanted into the right flank of the mice. After the transplantation, the size of each tumor was measured using calipers. Tumor volume (V) was calculated according to the formula

\[ V = a \times b^2 \times \frac{1}{2} \text{ (mm}^3\text{)} \]

where \(a\) and \(b\) being the tumor length and width (in mm), respectively.

The changes in relative tumor volume (RV):

\[ RV = \frac{V_n}{V_0} \]

where \(V_n\) is the tumor volume at day \(n\) and \(V_0\) is the initial volume immediately before the treatment was started (day 0). The mice were observed for 3 weeks after treatment because of pursuing tumor growth inhibition periods.

Evaluation: On any experimental day, T/C (%) was expressed as the average RV of the treated group vs that of controls. The effectiveness of each drug was evaluated on day 14 by the statistical significance of RV values between both groups determined by the Mann-Whitney’s U-test (P<0.01, one-sided) as well as the T/C (%) values, the effective criterion for which was 50% or less. The minimum dose that was shown to be effective for each drug was regarded as the minimum effective dose (MED) for mice.

Comparison of area under the curve (AUC) in dogs and mice: Murine and canine pharmacokinetic parameters [6, 8, 9, 12, 14–17, 24], and canine clinical doses (CDs) [24] of all antitumor agents were obtained from published references. The AUCs in canine CD and murine MED were calculated based on these data. Especially the CDs in DXR and CDDP were used for the dose of a small dog (<10 kg) [21]. As the antitumor effects of drugs having cell cycle non-specific activity are shown to be dependent on the AUC [27], the AUC of a dog given CD (AUC_dog)/AUC of a mouse given MED (AUC_mice) ratio was calculated. Drugs showing more than 1.0 in AUC_dog/AUC_mice ratio were regarded as effective in the patient. Clinical effects of the other drugs were predicted by comparison with AUC between canine CD and murine MED, and other pharmacokinetic parameters. The AUCs in CDDP and VLB were analyzed by protein-unbinding drug concentrations. And the AUCs in DXR, VCR and 5-FU were by total (protein-unbinding and binding) drug concentrations. In the case of CPM, the AUC was compared with 4-hydroxy CPM as an active metabolite between a mouse and a dog.

Combination chemotherapy: From the results of the single agent therapy, we had the experimental chemotherapy combined into two of the most effective agents, CDDP and CPM. CMG-6 was treated with the clinically equivalent dose (CED) of CDDP (6 mg/kg) and CPM (145.5 mg/kg). The CED indicates that the murine dose is a match for the murine AUC value that is equal to AUC_dog.

Histopathological examination: At the termination of these experiments, the mice were sacrificed and necropsied. Tumors and remarkable lesions in the mice were fixed in 10% neutral-buffered formalin and embedded in paraffin. Sections, 4 µm in thickness, were stained with hematoxylin and eosin.

RESULTS

Determination of MEDs in mice: The changes in relative growth rates (T/C %) of CMG-6 to six antitumor agents are plotted in Fig. 1. Profiles of experimental chemotherapy against CMG-6 are summarized in Table 1.

The MEDs (mg/kg) in mice were as follows; CPM 65 (MTD/4), DXR 6 (MTD/2), CDDP 5 (MTD/2), VCR 1.6 (MTD), VLB more than 5.5 (>MTD/2), 5-FU 105 (MTD/2).

DXR in 12 mg/kg (MTD), VLB in 11 and 8.25 mg/kg (MTD and 3MTD/4), and 5-FU in 210 mg/kg (MTD) was shown to be toxic in SCID mice. As VLB in 5.5 mg/kg (MTD/2) was ineffective against CMG-6, the MED of VLB could not be determined. Maximum body weight losses were indicated between 4 and 7 days after administration in some groups of drugs, however they gradually returned by the termination of the experiments. All mice in the control groups did not show any body weight loss or toxic death.

AUC_dog/AUC_mice ratios: AUC_dog/AUC_mice ratios are presented in Table 2. Drugs obtaining more than 1.0 in AUC_dog/AUC_mice ratio were CPM, CDDP, and 5-FU. The AUC_dog/AUC_mice ratios of DXR and VCR were less than 1.0, and that of VLB was less than 1.241.

Effect of combination chemotherapy: Growth rates in the experiment of combination chemotherapy are shown in Fig. 2. The T/Cs (%) of day 14 were 29 in CDDP, 25 in CPM and 5 in CDDP + CPM, respectively. All three groups were effective against CMG-6 according to the evaluation criteria of single agent therapy. Especially, tumor regression in the combination group was remarkable in the whole course of the experiment but these tumor nodules didn’t disappear. No mice showed toxic death in any group of this experiment.

Histopathological examination: There were no specific
CHEMOTHERAPY AGAINST CANINE MAMMARY CANCER

histological changes of tumor cells treated with antitumor agents. No lesions by toxicity of antitumor drugs or metastatic lesions were observed in autopsy. The mice that died by toxicity couldn’t be autopsied due to their post-morten changes.

DISCUSSION

Tumor xenograft systems have been widely used in human medical research, deeply contributing to the advancement of medical oncology [20, 29, 30]. As the recipients of xenografts, SCID mice deficient in both T and B cell functions [1] used in this experiment are better than nude mice deficient in T cell function [28] in the point of take rate, growth speed and metastatic behavior [13, 23, 38]. Also, the sensitivities of antitumor drugs against xenografts in SCID mice are reported to be only a little different from those in nude mice [39]. Compared with in vitro systems, the most important merit of tumor xenograft system is what is predicted the clinical effects of antitumor agents, especially using the clinically equivalent dose [17].

The chemosensitivities of CMG-6 showed a dose-dependent response for most of the antitumor agents. To predict the effectiveness in the canine patient from these results using the SCID mouse/canine tumor xenograft system, it is necessary to compare the drug pharmacokinetics between a mouse and a dog. The antitumor effects of drugs are determined by a protein-unbinding drug concentration in the tumor tissue, and the concentration is nearly equal to a venous plasma protein-unbinding one [34]. The antitumor effects of drugs having cell cycle non-specific activity (CCNS, Type I) such as CPM, DXR and CDDP are proven to be dependent on the AUC [27, 32]. On the other hand, the effects of some drugs having cell cycle specific activity (CCS, Type II) including VCR, VLB, and 5-FU are now unable to be determined by any pharmacokinetic parameters of these drugs. Therefore, these clinical antitumor effects were evaluated by the AUCs and other pharmacokinetic parameters in comparison between mice and dogs [26, 31, 33, 37].

In the case of CCNS drugs, murine doses of canine CEDs were calculated from the AUC/MED ratios; CPM 145.5 mg/kg, DXR 1.1 mg/kg and CDDP 6 mg/kg. In DXR and CDDP, lower doses were used from the canine patient’s body weight (<10 kg). Therefore, the AUC/MED ratios of these two drugs are thought to be higher in dogs more than 10 kg.

In the case of CCS drugs, protein binding of VCR is slight in both mice and dogs, therefore the AUC was evaluated by total concentration. Murine MED was high (MTD) and the
AUC ratio was very low (0.040). VCR would not be effective in the patient, however, evaluation of only one parameter as the AUC value might not be reasonable for the prediction of clinical effects of VCR. MED of VLB could not be determined by toxic death in MTD and 3MTD/4. If MED of VLB might be determined as 3MTD/4 or MTD, the AUC ratio would be 0.828 or 0.621, respectively. From this point of view, VLB would not be effective in the patient. AUC ratio was 1.147 and 5-FU would be effective in the patient. But 5-FU is a Type II drug and it might not be accurate in predicting the clinical effect by the total AUC.

Table 1. Profiles of experimental chemotherapy against canine mammary gland tumor xenograft (CMG-6)

<table>
<thead>
<tr>
<th>Drug</th>
<th>Dose (mg/kg)</th>
<th>T/C (%) in Day 14</th>
<th>Rate of Death in Treatment Group</th>
<th>Maximum Body Weight Loss (%) [day]</th>
<th>Growth Rate of Control in Day 14</th>
<th>Number of Mice in Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPM</td>
<td>260</td>
<td>4*</td>
<td>0/8</td>
<td>14[4]</td>
<td>7.63</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td>130</td>
<td>11*</td>
<td>0/6</td>
<td>8[4]</td>
<td>6.74</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>65 (a)</td>
<td>25.2*</td>
<td>0/6</td>
<td>4[4]</td>
<td>8.56</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>32.5</td>
<td>82</td>
<td>0/6</td>
<td>0</td>
<td>7.74</td>
<td>7</td>
</tr>
<tr>
<td>DXR</td>
<td>12</td>
<td>8/9 of mice died by day 10.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>6 (a)</td>
<td>34*</td>
<td>0/6</td>
<td>18[7]</td>
<td>6.05</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>74</td>
<td>0/6</td>
<td>5[4]</td>
<td>6.74</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>1.5</td>
<td>104</td>
<td>0/5</td>
<td>2[4]</td>
<td>6.74</td>
<td>5</td>
</tr>
<tr>
<td>CDDP</td>
<td>10</td>
<td>Not tested.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5 (a)</td>
<td>33*</td>
<td>0/6</td>
<td>2[4]</td>
<td>8.05</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>2.5</td>
<td>76</td>
<td>0/6</td>
<td>0</td>
<td>8.05</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>1.25</td>
<td>83</td>
<td>0/6</td>
<td>0</td>
<td>8.05</td>
<td>6</td>
</tr>
<tr>
<td>VCR</td>
<td>1.6 (a)</td>
<td>49*</td>
<td>0/6</td>
<td>13[4]</td>
<td>8.41</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>0.8</td>
<td>71</td>
<td>0/6</td>
<td>0[4]</td>
<td>8.41</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>0.4</td>
<td>88</td>
<td>0/6</td>
<td>0</td>
<td>8.41</td>
<td>7</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
<td>108</td>
<td>0/7</td>
<td>0</td>
<td>8.41</td>
<td>7</td>
</tr>
<tr>
<td>VLB</td>
<td>11</td>
<td>5/7 of mice died by day 14.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>8.25</td>
<td>Not tested.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>5.5</td>
<td>78</td>
<td>3/7</td>
<td>0</td>
<td>6.08</td>
<td>7</td>
</tr>
<tr>
<td>5-FU</td>
<td>210</td>
<td>4/7 of mice died by day 14.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>105 (a)</td>
<td>41*</td>
<td>2/6</td>
<td>6[7]</td>
<td>6.68</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>52.5</td>
<td>93</td>
<td>1/6</td>
<td>0</td>
<td>6.68</td>
<td>6</td>
</tr>
</tbody>
</table>

*a*: Statistical significance by the Mann-Whitney’s U-test (P<0.01, one sided). a) Minimum effective dose (MED).

Table 2. Prediction of clinical effects of six antitumor agents in the patient with canine mammary gland tumor (CMG-6)

<table>
<thead>
<tr>
<th>Drug</th>
<th>MED (mg/kg)</th>
<th>AUCmouse (µg hr/ml)</th>
<th>CD (mg/kg)</th>
<th>AUCdog (µg hr/ml)</th>
<th>AUCdog/AUCmouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>CPM</td>
<td>65</td>
<td>6.54[15]</td>
<td>11.7 (250 mg/m²)</td>
<td>14.64 [9]</td>
<td>2.239</td>
</tr>
<tr>
<td>DXR</td>
<td>6</td>
<td>2.26[13]</td>
<td>1</td>
<td>0.42 [14]</td>
<td>0.186</td>
</tr>
<tr>
<td>CDDP</td>
<td>5</td>
<td>1.28[23]</td>
<td>2.3 (50 mg/m²)</td>
<td>1.53[16]</td>
<td>1.200</td>
</tr>
<tr>
<td>VCR</td>
<td>1.6</td>
<td>0.99[15]</td>
<td>0.033 (0.7 mg/m²)</td>
<td>0.04 [14]</td>
<td>0.040</td>
</tr>
<tr>
<td>VLB</td>
<td>&gt;5.5</td>
<td>&gt;0.29[15]</td>
<td>0.094 (2 mg/m²)</td>
<td>0.36[6]</td>
<td>&lt;1.241</td>
</tr>
</tbody>
</table>

a) AUC of mice given MED. b) AUC of dogs given CD. c) free AUC (AUC in unbound drug concentration with plasma proteins).
There are many advantages in the use of combination chemotherapy. The fraction of tumor cells killed by one chemotherapeutic drug is independent of that killed by another. Drugs can be used in combination to attack the different fractions of the tumor cells. We can choose drugs that have different major toxicities in order to limit any one toxicity and to allow each drug to be used in full dosage [4, 7]. Combination chemotherapy also helps to avoid both inherent drug resistance and the emergence of resistant subpopulations due to acquired resistance [5]. In the combination chemotherapy using the CEDs in CDDP and CPM that have two of the highest values in AUCdog/AUCmouse ratios, the group given these two drugs showed the most effect of all the groups. In human medicine, adjuvant and combination chemotherapy that contained 5-FU, DXR, and CPM (FAC) [3] or CPM, methotrexate, and 5-FU (CMF) [35] is used as a treatment for breast cancer with or without radiation and/or hormonal therapy. However, the usefulness of combination chemotherapy against mammary cancers has not been sufficiently proven in veterinary medicine. Clinical trials of combination chemotherapy should be carried out to evaluate both the antitumor effects and the side effects.

From these results, we could detect clinically effective drugs in the patient with CMG-6. The combination chemotherapy of CDDP and CPM is also suggested to indicate the additional effect of the respective drugs. By the way, chemosensitivity of cancers originating from the same tissue and also with the same histology is known to be different. To apply for the clinical treatment protocols of canine mammary cancers based on the result, it is necessary to evolve the studies of several canine mammary cancer xenografts.

ACKNOWLEDGEMENTS. The author would like to thank Dr. Minoru Shimoda (Department of Veterinary Pharmacology, Faculty of Agriculture, Tokyo University of Agriculture and Technology) for advice of the interpretation of pharmacokinetic data, and Mr. Taichiro Sugii and Mr. Atsushi Okano (Department of Veterinary Surgery, Faculty of Agriculture, Tokyo University of Agriculture and Technology) for support in experiments.

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