Inhibitory Effects of Glucocorticoids on Proliferation of Canine Mast Cell Tumor

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ABSTRACT. The inhibitory effect of glucocorticoids (GCs) on proliferation of canine mast cell tumor (MCT) was studied using two types of MCT cells; JuMC cells and LuMC cells derived from spontaneous canine cutaneous and intestinal MCT, respectively. In vitro study, growth of JuMC cells was significantly inhibited with more than 1 nM GCs and apoptotic-like cell death was seen, while that of LuMC cells was not inhibited even with 10 μM GCs. Growth rate of mass in nude mice developed by inoculation of JuMC cells was reduced in a dose-dependent manner by administration of GC, while growth inhibition of masses developed by inoculation of LuMC cells was minimal with increasing GC dose. Competitive binding studies and Scatchard analysis demonstrated the presence of high-affinity, low capacity GC receptors in both JuMC and LuMC cells. Kd was estimated to be 1.30 nM for JuMC cells and 0.45 nM in LuMC cells, respectively. It is concluded that canine cutaneous MCT cells responded to GCs in vitro and in vivo, whereas intestinal MCT cells did not, though both types of cells had specific GC receptors. — KEY WORDS: canine, cell line, glucocorticoid, glucocorticoid receptor, mastocytoma.

Mast cell tumor (MCT) accounts for 7 to 21% of all skin tumors and 11 to 27% of all malignant skin tumors in dogs [9, 23]. Canine MCT occurs primarily in the dermis and subcutaneous tissues, which is called as cutaneous MCT. Mast cell tumor cells have cytoplasmic granules containing several vasoactive substances like histamine and heparin, which often cause various clinical symptoms such as gastric ulcer and bleeding tendency in the tumor patients. Surgical excision is the choice of treatment for cutaneous MCT, however it often metastasizes to the adjacent and/or distant skin and visceral lymph nodes. In addition, the vasoactive substances of this tumor affect the systemic conditions.

Another MCT, called as intestinal MCT, is derived from the mast cells in the mucosal or submucosal tissues of the intestines, however the reports on this type of MCT in dogs are few [18]. Intestinal MCT rarely responds to various treatments including surgery and chemotherapy. The median survival time of intestinal MCT patients in Veterinary Medical Center of The University of Tokyo does not exceed a month, whereas that of cutaneous MCT is from 5 months to 24 months according to the malignancy of the tumor (data not shown).

For cutaneous MCT, adjunctive chemotherapy with glucocorticoids (GCs), vincristine and/or cyclophosphamide combined with surgical resection and/or radiotherapy often applied [16]. Glucocorticoid is one of the agents that have been proved to be effective on cutaneous MCT [5, 17], however the precise mechanism of the inhibitory effect has not been clarified. The inhibitory effect of GC on the lymphoma is also widely recognized [15]. Lymphoma cells are known to possess the GC receptor [10], which may mediate the inhibitory effect of GC on lymphoma cell. Mast cell is also derived from hematopoietic stem cells.

We have established both cutaneous and intestinal types of canine MCT cells from the spontaneous canine cases [18, 19]. In this experiment, in vitro and in vivo inhibitory effects of GCs on these tumor cells were investigated and cytoplasmic GC receptors of these cells were measured.

MATERIALS AND METHODS

MCT cells and culture method: JuMC cell was derived from spontaneous canine cutaneous MCT clinically categorized as intermediately-differentiated MCT, which had been treated with prednisolone (PRD) as one of the chemotherapeutic agents. This cell was maintained in our laboratory by in vitro passage (subcutaneous inoculation) in female nude mice (5 week old, BALB/c-nu/nu Slc, Nippon SLC Inc., Shizuoka, Japan) in every 6 to 10 weeks. This cell was characterized as rat connective tissue type mast cell by morphology and the manner of degranulation by various stimulations [19]. Doubling time of this cell in vitro was over 300 hr. In this study JuMC cells with 20 to 24 passages were used.

JuMC cells were obtained by excision of the mass from the nude mouse. The mass was minced in phosphate buffered saline (PBS(−)), filtrated through a 53-μm mesh, washed and centrifuged at 1,000 rpm for 5 min. Then it was resuspended and cultured in RPMI-1640 medium (2 mM L-glutamine, 10 U/ml penicillin, 10 μg/ml streptomycin, 50 μM 2-mercaptoethanol) with 10% heat-inactivated fetal bovine serum (FBS) (JRH Biosciences, U.S.A.) at 37°C in humidified air with 5% CO2.

LuMC cells were derived from spontaneous canine intestinal MCT. The case also had a history of receiving PRD treatment for indistinct eosinophilia. This cell was
established as a cell line in our laboratory. The character of this cell resembles rat basophilic leukemia cell line (RBL-2H3) [18], which is an analogue of mucosal type mast cell in morphology and in the manner of degranulation. Doubling time at a starting concentration of $5 \times 10^5$ is about 24 hr. In this study LuMC cells with approximately 180 passages were used.

LuMC cells were cultured in RPMI-1640 medium (2 mM L-glutamine, 50 µg/ml gentamicin, 1.5 µg/ml fungisone, 4.4 µg/ml lipoprotein) with 10% heat-inactivated FBS at 37°C in humidified air with 5% CO$_2$.

*Changes in cell number by addition of GCs:* JuMC cells ($5 \times 10^5$/ml) and LuMC cells ($2 \times 10^5$/ml) in log phase of growth were evaluated in the above media in duplicate with or without water soluble dexamethasone (DEX) (Sigma Chemical Co., St. Louis, U.S.A.) or PRD 21-hemisuccinate (Sigma). The final concentrations of both drugs were 0.1, 1, 10, 100 nM, 1 and 10 µM. After incubation for 1, 2, 3, 4, 5 and 6 days for JuMC cells and 1, 2, 3 and 4 days for LuMC cells, respectively, the total number of viable cells was counted by trypan-blue dye exclusion method.

*MTT assay:* Inhibitory effects of GCs on the metabolic activity of these cells were evaluated by MTT assay. The principle of MTT assay is based on the cellular reduction of MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) by mitochondrial dehydrogenase of viable cells to a blue formazan product that can be measured spectrophotometrically [6]. Briefly, triplicate suspensions of JuMC cells ($5 \times 10^5$/100 µl) and LuMC cells ($2 \times 10^5$/100 µl) were incubated in 96-well microculture plates with or without GCs for 48 hr. The final concentrations of water soluble DEX or PRD 21-hemisuccinate was 0.1, 1, 10, 100 nM, 1 and 10 µM. After centrifugation of the cell suspension at 2,000 rpm for 5 min, the supernatant was discarded and 10 µl of 0.4% MTT (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and 10 µl of 0.1 M sodium succinate (Wako) were added to each well. After incubation for 3 hr at 37°C, 150 µl of dimethyl sulfoxide (Wako) was added as a solvent. After another incubation for 10 min, optical density (OD) at 540 nm was measured by microplate spectrophotometer (Bio-Rad Laboratories, Inc., Tokyo, Japan) with 630 nm as the reference wavelength. Inhibition index (I,I) was calculated by the following equation: I,I=([a-p]/(a-m))×100 (%) (a=OD of control well, p=OD of treated well, m=OD of background).

*Cell morphology:* JuMC cells ($5 \times 10^5$/ml) and LuMC cells ($2 \times 10^5$/ml) were incubated in the above media with or without water soluble DEX at final concentrations of 1 nM and 1 µM. After incubation for 2 or 6 days, aliquot of the cells was centrifuged and the medium was aspirated. Then cells were fixed in Carnoy’s solution, applied to slides and stained with May-Giemsa solution. Staining with 0.1% toluidine blue at pH 0.3 for JuMC cells and 0.02% azure A at pH 0.5 for LuMC cells, respectively, was also performed to identify the mast cell specific granules [7, 8].

*In vivo effects of GC on MCT cell growth in nude mice:* In vivo inhibitory effects of PRD on the growth of MCT cells inoculated into nude mice were evaluated according to the protocol of Battle Columbus Laboratories [11], which was proposed for screening of antitumor drugs using nude mice.

Both cultured cells were washed in PBS (−) and prepared for the inoculation as $1 \times 10^7$ cells per 300 µl PBS (−). The cell suspensions were inoculated subcutaneously on the back of the nude mice at 5 weeks of age with a 25 G needle, which had been irradiated (4 Gy) 3 days before the inoculation. The mice were housed in isolated cages (isolack©, ICM Inc., Ibaraki, Japan) and fed γ-ray irradiated food and autoclaved-water ad libitum.

Twenty-two to 36 days after JuMC cell inoculation, when the tumor mass developed to an estimated volume of 100–300 mm$^3$, nude mice were divided into 4 groups (each group consisted of 6 mice) as follows. Those of group T were intraperitoneally administered PRD sodium succinate (Predonine®, Shionogi, Osaka, Japan) at a dose of 2, 20 or 200 mg/kg (in 200 µl solution) or 21 days. Those of control group (group C) were received the same volume of physiological saline solution for the same period. Tumor size was measured every third day with a sliding caliper in two dimensions. The tumor volume was estimated by the following equation: $V=L \times W^2/2$ (L=maximum diameter, W=diameетer at right angles to the length). On day 21 mice were euthanatized, body weight was measured, and tumor masses were excised. The masses were weighed and fixed in phosphate-buffered formaline or Carnoy’s solution, paraffin-embedded and stained with hematoxylin-eosin or 0.1% toluidine blue at pH 0.3 [7, 8]. The effect of PRD was expressed as the tumor growth inhibition rate (IR), calculated by the following formula using the average weight of tumor mass of the groups T and C at the end of the treatment: IR=(1−T/C)×100(%)..

In nude mice inoculated LuMC cells, PRD was administered at the same doses as in group T (each group consisted of 4 mice). Prednisolone treatment was initiated after 24 hr post-inoculation, because it was expected from preliminary experiment that the tumor mass grew quite fast but became eroded and infiltrated into the surrounding tissue. Tumor size could not be measured because of the diffuse shape of the mass developed. Therefore, inhibitory effects were evaluated by IR alone.

*GC receptors in MCT cells:* The binding of GC to specific high affinity cytosol receptors of both JuMC and LuMC cells was determined by the competitive binding assay developed by Baxter and Tomkins using radioactivity-labeled and -unlabeled DEX [3]. Linear least-squares regression analysis of the binding data was used to estimate total saturable binding sites and apparent dissociation constant ($K_d$) according to the method of Scatchard [25].

*Statistical analysis:* For the comparisons of changes in cell number, I,I, estimated tumor volume and tumor weight between different groups, a one-factor ANOVA followed by a Scheffé’s multiple comparison test was used. $P$ values less than 0.05 were considered as statistically significant.
RESULTS

Changes in cell number: Changes in JuMC cell number when DEX or PRD was added to the culture media were shown in Fig. 1 (upper panel). In JuMC cells after incubation with DEX, the cell number was not significantly changed for 3 days. However 4 days after the addition, cells treated with more than 1 nM DEX showed a significant reduction in number compared to the control group, and tended to reduce thereafter. With 0.1 nM DEX, cell growth was slightly inhibited but there was no significant difference in cell number from that of control group. Similarly, while 0.1 nM PRD did not affect JuMC cell growth, more than 1 nM PRD significantly inhibited JuMC cell growth compared to the control group.

On the contrary, these GCs never inhibited LuMC cell growth in vitro (Fig. 1, lower panel). Though growth of treated cells tended to be slightly inhibited, there was no significant difference in cell number compared to the control group. The cell number of any groups treated with DEX or PRD showed the similar increasing patterns during the experimental period.

MTT assay: Dexamethasone and PRD at any doses also showed strong inhibition of JuMC cell activity (Fig. 2). There was no significant difference in the inhibitory effect between concentrations of GCs. In even the lowest dose of both drugs (0.1 nM), I.I. values of DEX-treated and PRD-treated JuMC cells were 35.4 ± 2.0% (mean ± SD) and 32.9 ± 3.1% (mean ± SD), respectively. The degree of inhibition was slightly milder in PRD-treated cells than in DEX-treated cells, but there was no significant difference in the inhibitory effect between both drugs.

In LuMC cells, I.I. did not exceed 15% at any doses (Fig. 2). In even the highest dose of GCs (10 μM), I.I. values of DEX-treated and PRD-treated cells were 13.9 ± 7.8% (mean ± SD) and 8.3 ± 5.8% (mean ± SD), respectively. Dexamethasone seems to be more effective than PRD as in JuMC cells.

Cell morphology: In JuMC cells after 2 days of incubation with more than 1 nM GCs, phase-contrast microscopy revealed that most of the cells became smaller in size and that some of the cells were stained with trypan blue, indicating cell death. The number of trypan blue stain-positive cells increased according to the increase in incubation period. At the dose of 0.1 nM GC, morphological changes were not observed.

Fig. 1. Effects of dexamethasone (DEX) and prednisolone (PRD) on the growth of mast cell tumor cells (upper panel: JuMC cells; lower panel: LuMC cells). Viable cells were counted daily with trypan blue staining. * P<0.05 compared with control; # P<0.05 compared with 10⁻¹⁰ M.
In LuMC cells, more than 95% of GCs-treated or non-treated cells were not stained with trypan blue during the experimental period.

Light microscopic findings of JuMC cells after incubation for 2 days with DEX at 1 μM were shown in Fig. 3. In JuMC cells, the treated cells were apparently smaller and some of the cells had several fragmentations of pyknotic nuclear materials, which grew similar findings to those seen in apoptotic cells [26]. There was no significant difference in morphology between both doses of DEX. The changes in metachromatic granules were not detected in JuMC cells treated with both doses of DEX.

LuMC cells treated with DEX did not reveal any morphological changes. Mitoses were observed even in the cells treated with 1 μM DEX.

**Growth inhibition of PRD on MCT cells in vivo:**
Inhibitory effect of PRD on JuMC cells inoculated into nude mice are shown in Fig. 4. Masses formed by inoculation continued to grow after administration of PRD, however the growth rates were reduced in a dose-dependent manner. In any of group T, significant difference in growth rate was detected compared to the group C. Even 2 mg/kg of PRD, the lowest dose, could significantly reduce the growth rate of the mass. Final tumor weight also represents significant inhibition of PRD at any doses compared to group C (Table 1). Tumor growth inhibition rates on day 21 at doses of 2, 20, 200 mg/kg were 35, 50, 73%, respectively.

In LuMC cell, growth rates seemed to decrease with increasing PRD doses, though there was no significant difference in tumor weight at necropsy from those of group C (Table 1).

Histological examination of the tumor mass at necropsy revealed no significant differences between the groups and no apoptotic changes were observed.

**GC receptors in MCT cells:** Competitive binding studies and Scatchard analysis demonstrated the presence of high-affinity, low-capacity GC receptors in both JuMC and LuMC cells (Fig. 5). Saturation of the receptor was archived at low GC concentrations and K_d was estimated to be 1.30 ± 0.21 nM (mean ± SD) in JuMC cell and 0.45 ± 0.11 nM (mean ± SD) in LuMC cell, respectively. The number of binding sites was 67.5 ± 28.0 pmol/mg cytosol protein (mean ± SD) in JuMC cell and 68.2 ± 5.7 pmol/mg cytosol protein (mean ± SD) in LuMC cell, respectively, and only one class of binding sites was revealed.

**DISCUSSION**
Well-differentiated or immediately-differentiated canine cutaneous MCT often responds to GC therapy. Through our experiences, GC seems to have equal or more inhibitory effects on the tumor growth than antineoplastic agents such as cyclophosphamide and vincristine [5, 20].

In this study, it was demonstrated that JuMC cells had the specific cytoplasmic receptor to GC and that their growth was markedly inhibited by GC treatment both in vitro and in vivo. On the contrary, LuMC cells did not show any
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significant reduction in growth by GC treatment in vitro and in vivo, though they also had the specific receptor to GC. Since mild but significant inhibition of LuMC cell activity was observed by GC treatment in MTT assay, LuMC cell growth might be inhibited by GC to a certain

Fig. 4. Effect of prednisolone (PRD) on growth of JuMC cells inoculated into nude mice. *: $P<0.05$ compared with control; †: $P<0.05$ compared with PRD 2 mg/kg group. Data are expressed as mean ± SEM.

Fig. 5. Typical binding curves of [3H] dexamethasone to JuMC cells (upper panel) and LuMC cells (lower panel). The binding data is replotted in the inset according to the Scatchard technique. "r" is the correlation coefficient for each line.
extent, but the damage of the cells might be minimal to cause reduction in cell number.

Decreased number of cultured JuMC cells was attributed to cell death. Morphologically, reduction in cell size and fragmentation of pyknotic nuclear materials were observed, which is the characteristic feature suggesting apoptotic cell death [26], though DNA fragmentation must be proved for the definition of apoptosis.

The action of GC is known to be mediated through their interaction with GC receptors in the cytosol and nuclear fraction [3]. In lymphoid cells, GC-induced cytolysis is mediated through GC receptors [2]. Since the origin of mast cell is hematopoietic stem cell, cytolethal effect of GC on mast cells might be mediated by the similar mechanism. In this study, there was an apparent difference in sensitivity to GC between JuMC and LuMC cells. Ligand-binding ability of the receptor of both cells was proved to be intact. The number of binding sites was almost equal in both cells, and interestingly, ligand-binding affinity of the receptor of LuMC cells was three times as high as that of JuMC cells. In JuMC cells, cell growth in vitro was inhibited within the range of calculated $K_d$ (1.3 nM), in which dose a half of receptors is saturated with GC. Though sensitivity of LuMC cells to GC must be higher than that of JuMC cells, LuMC cells were resistant to the cytolethal effect of GCs. The correlation between receptor binding affinity and reaction in vitro was not found in LuMC cells.

Generally, it is thought that poorly differentiated MCT cells may lose GC receptors and therefore may be resistant to GC therapy [23], or that tumor-resistant clones with fewer or ineffective GC receptors may grow [16]. Relationships between receptors and reaction of tumor cells in vitro or in vivo have been well investigated in lymphoid cells. Studies using mouse lymphoma cell lines have demonstrated a fairly good correlation between the level of GC receptor and the in vitro action of steroids [24]. Conversely, no correlation was observed between the level of GC binding and cytotoxic or cyostatic responsiveness of human lymphoid cell lines to GC treatment in vitro, and some of the insensitive cell lines exhibited the highest levels of steroid binding [1]. Authors concluded it unable to detect any significant quantitative differences in cytosol or nuclear steroid binding properties of different types of human leukemic cell lines. These reports suggest that the presence of GC receptors does not always mean the responsiveness of the cell to GC treatment, like the results of this study.

It has recently been shown that the resistance is partially due to mutations in GC receptors. In GC receptors from GC-resistant human leukemic cells, point mutations abolished transcriptional activation without any apparent alteration in the ligand-binding properties or nuclear translocation of ligand-receptor complexes [21]. Glucocorticoid receptors of LuMC cells may be also intact in ligand-binding characteristics but be deficient in other functions. Other potential mechanisms that induce GC-resistance of LuMC cells might be overexpression of the plasma membrane P-glycoprotein [4] and abnormal heat shock proteins expression [14]. Some forms of steroid resistance may have developed during the long period of cultivation of LuMC cells in vitro, or initially it may have had GC-resistant characteristics.

In in vivo study, the schedule and the route for drug administration were decided with reference to the clinical treatment, and to the experiment where the dose in the present study was thought to be a maximum tolerated dose for mice. In addition, only PRD was used as GC, since the in vitro study suggested DEX and PRD have the similar inhibitory effects on cell growth.

Prednisolone dose-dependently inhibited growth of the mass developed in nude mice inoculated with JuMC cells. There have been few reports on the mechanism of the in vivo effect of GCs on MCT cells in dogs. Development of connective tissue type mast cell is thought mainly to depend on stem cell factor (SCF) derived from fibroblasts [13]. In mice, connective tissue type mast cells die rapidly after withdrawal of SCF in vivo or in vitro, and they undergo apoptosis [12]. JuMC cells are derived from cutaneous MCT and their character suggests them as connective tissue type mast cells, thus the inhibitory effect of GC on JuMC cell in vivo may be mild under the presence of fibroblasts. In contrast, interleukins-3 and -4 produced by T cells are thought to control differentiation and proliferation of normal mast cells, especially of mucosal type mast cell in rodents [22, 27]. Regulation of T cell-produced cytokines by GC may be attributable to diminished mucosal type mast cell proliferation during administration of GC in vivo. In this study, slight reduction in size of tumor mass in T cell deficient nude mice may represent the direct interaction with GC and LuMC cells, which is categorized as mucosal type mast cell, not associated with T cell.

It is concluded that canine cutaneous MCT cells responded to GCs in vitro and in vivo, whereas intestinal MCT cells did not, though both types of cells had specific GC receptors. Further studies will be needed to clarify the discrepancy between the presence of receptors and different responses in both types of cells to establish a better treatment modality for canine MCT.

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