論 文 の 内 容 の 要 旨

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論文題目　Effects of metronomic chemotherapy using cyclophosphamide and piroxicam in canine oral malignant melanoma
（犬口腔内悪性黒色腫に対するシクロフォスファミドとピロキシカム併用メトロノーム化学療法の有用性に関する研究）

Malignant melanoma (MM) is one of the most common oral malignant tumors in dogs which accounts for 30 to 40% of all canine oral tumors. Biological behavior of the canine oral MM is highly malignant with invasion of surrounding tissue and the metastasis rate is reported in up to 80%. Although several treatments including surgery, radiation therapy and conventional chemotherapy have been attempted, survival time in most dogs accompanied with oral MM is less than one year.

Recently, metronomic chemotherapy has been reported as a promising alternative treatment mainly for an adjuvant therapy after surgical resection of tumors in both human and canine cancer patients. Metronomic chemotherapy generally refers to low dose of chemotherapy drugs, such as cyclophosphamide, at short intervals designed to minimize toxicity and target the endothelium or tumor stroma instead of targeting the tumor by maximum tolerated dose (MTD) therapy. Metronomic chemotherapy has been reported to show the superior outcome as a “tumor dormancy” therapy by suppressing the progression of malignant disease and improving the quality of life.

One of the major anti-tumor mechanisms of metronomic chemotherapy is supposed to be an anti-angiogenesis effect based on the endothelial cytotoxicity and the induction of anti-angiogenic protein, Thrombospondin-1 (TSP-1). TSP-1 modulates anti-angiogenesis by binding and displacing of pro-angiogenic factor, the vascular endothelial growth factor (VEGF). Canine oral MM has been reported to show the high expressions of the cyclooxygenase-2 (COX-2) and VEGF. Non-Steroidal Anti-Inflammatory Drugs (NSAIDs) inhibit the COX-2 activity from converting arachidonic acid into prostaglandins (PGs) family, such as PGE2, and the reduction of PGE2 causes the decrease of VEGF expression. Thus NSAIDs is expected to show the

same or synergistic effect as the combination drug for metronomic chemotherapy. Piroxicam, one of the NSAIDs is used for inflammatory diseases routinely and applied for some tumors as the anti-tumor drug by its inhibitory effect against COX-2 in veterinary medicine. Therefore, the combination of metronomic cyclophosphamide and piroxicam might be a potent protocol in these aspects.

The purpose of this study was to investigate the effectiveness of metronomic chemotherapy using low-dose cyclophosphamide and piroxicam in canine oral MM by means of the anti-angiogenesis, anti-proliferation, induction of tumor cell apoptosis *in vitro* and *in vivo*. In addition, the clinicopathological features and the clinical effectiveness of metronomic chemotherapy on the survival of canine oral MM patients were elucidated.

In Chapter 1, anti-tumor effects of metronomic cyclophoshamide, piroxicam and their combination on canine oral MM cell lines were evaluated. First, the expressions of COX-2 in six canine MM cell lines, CMeC-1, CMeC-2, LMeC, KMeC, CMM1 and CMM2 were preliminary evaluated. Among those cell lines, high expression of COX-2 was detected in CMM1 which was established from MM of clinical stage III. Therefore, CMM1 was used in the following experiments. Treatment doses used in this *in vitro* study were determined by the results of MTT assay of CMM1; the highest non-toxic dose was used for low-dose cyclophosphamide dose and IC 50 dose for piroxicam dose. Treatment regimens were designed as follows; no drug (control), low-dose cyclophosphamide alone (CyLD), piroxicam (Px) alone and the combination of low-dose cyclophosphamide and piroxicam (CyPx). Anti-tumor effects of these regimens on CMM1 cells were investigated *in vitro* as follows; cell numbers was evaluated by Trypan blue exclusion assay, morphological appearance by phase contrast microscopy, cell viability by MTT assay, proliferative index (PI) by the Ki-67 immunostaining, PGE2 production by ELISA, expressions of pro- and anti-angiogenic proteins VEGF and TSP-1, anti-apoptotic protein Bcl-2 and tumor activity markers ERK and phosphorylated-ERK (p-ERK) by western blotting. CMM1 cell numbers were comparable during 0-12 hours, slightly different at 24 hours and significantly different at 48 hours between with (Px and CyPx groups) and without (control and CyLD groups) piroxicam treatment. The similar results were observed in PI and cell viability at 48 hours of incubation. In addition, CMM1 cell showed less viability in the CyPx group than that of the Px group. PGE2 production revealed significant difference between with and without the piroxicam treatment. Regarding to angiogenic factors, TSP-1 decreased chronologically in CyLD and CyPx groups, while VEGF did not change in all treatment regimens. Anti-apoptotic factor, Bcl-2 was chronologically decreased in the treatments that used piroxicam. For the proliferative factor, all treatments chronologically decreased the expression of p-ERK, particularly the Px group. Form the results of *in vitro* study, the anti-tumor effect of these regimens was observed in the aspect of anti-proliferative activity, however the anti-angiogenic effect was still controversial.

In Chapter 2, the anti-tumor effect of each regimen was investigated in *in vivo* study. To establish the suitable model which simulates the tumor microenvironment and to evaluate the effect of metronomic chemotherapy *in vivo*, the time course of tumor progression in the aspects of cell proliferation and angiogenesis in the CMM1 cells transplanted mouse model was evaluated. After transplantation of CMM1 cells

subcutaneously into the back of the nude mice, primary tumor masses enlarged gradually Although the VEGF expression which was detected by immunohistochemistry was comparable among all the time points, tumor neovascularization which was detected by MVD value on primary mass progressively increased during 10-20 to 20-30 days, and then reached a plateau during 30 and 40 days. Protein expressions by western blot analysis, COX-2 showed highest expression at 10 days, VEGF was maintained at same levels in each time point. Although ERK was maintained at similar levels, p-ERK highly expressed during the 20-30 days. Each treatment was started 10 days after transplantation of CMM1 at which time tumor was approximately 250 mm^2 in size. The CyLD group was given20 mg/kg/SID of cyclophosphamide mixing in the drinking water. The Px group was treated with 0.3 mg/kg/day mixed with sesami oil administered by oral gavage. The CyPx group was treated with both drugs. After treatment of 1 week, primary tumor masses in xenografted mice treated with CyLD and CyPx groups were significantly smaller than those of control and Px groups. At the end of experiment, in all treatment groups, especially in the CyPx group significant tumor growth inhibition was observed as compared with the control group. No severe side effect was observed in all treatment groups at any time point. PI was significantly decreased in all treatment groups; however the expression of p-ERK was low in Px and CyPx groups and high in the CyLD group. According to the anti-angiogenic effect, the degrees of tumor angiogenesis which detected by both MVD and VEGF expression, were lowered in all treatment groups, but the significant difference was detected only between the control and the CyPx groups. Double immunofluorescent staining with CD31 as the endothelial cell marker and α-smooth muscle actin (α-SMA) as the pericyte marker was performed to detect the vascular normalization. The percentage of vascular normalization, indicated by the number of double CD31/α-SMA positive vessel intotal vessel number, was significantly decreased only in the CyLD group. In addition imbalance of VEGF and TSP-1 which are angiogenic proteins detected by western blot analysis was observed only in this group. Tunnel assay was also perfomed on the tumor tissue to detect cell apoptosis, The number of apoptotic cells was significantly different between the treatment groups with and without piroxicam. Bcl-2, the anti-apoptotic protein detected by western blot analysis revealed low expression in Px and CyPx groups, but high expression in the CyLD group. The use of metronomic cyclophosphamide alone seems to have less anti-tumor effect, because of the pathogenic tumor vessels which induced by the imbalance of angiogenic proteins. The combination with piroxicam might have more potent anti-tumor effect on canine oral MM with anti angiogenic effect both in quality and in quantity. In addition, this regimen did not cause severe side effect in transplanted mice. The combination of metronomic cyclophosphamide and piroxicam was considered to be a useful protocol for canine oral MM patients.

In Chapter 3, clinical effectiveness of metronomic cyclophosphamide and piroxicam on spontaneous canine oral MM was evaluated. First, the proliferative and angiogenic profiles and their correlations to the clinicopathological features and prognostic outcome of canine oral MM cases were investigated retrospectively using medical records and phone interview. 37 dogs were used for investigation, and most canine oral MM was detected in small-breed dogs and their mean age was 11.7 ± 2.4 years. The number of the patients was comparable between male and female. Out of 42 MM specimens from 37 dogs, most primary MMs were less pigmentated. Primary tumor tissue was predominantly found at gingiva, followed by mucosa and palate. By

double immunofluorescent staining with Ki-67 for PI and CD31 for MVD, PI was significant correlated with the clinical stage, the degree of pigmentation, and the survival period. In contrast, the expression of MVD was found to have no correlations with clinical and histopathological features, including PI and the survival period. Second, prospective clinical trial of metronomic chemotherapy was performed in 6 dogs with oral MM. In this clinical trial, 12.5 mg/m^2/daily of cyclophosphamide and 0.3 mg/kg/daily of piroxicam were orally administrated as the metronomic chemotherapeutic protocol (CyPx). Treatments with or without metronomic chemotherapy were as follows; non-treatment (n=5), surgical excision (Sx, n=8), radiation therapy (Rx, n=6), surgical excision and radiation therapy (Sx+Rx, n=7), surgical excision and metronomic chemotherapy (Sx+CyPx, n=2) and surgical excision, radiation therapy and metronomic chemotherapy (Sx+Rx+CyPx, n=4), respectively. Among these treatment methods, survivals time of dogs treated with Sx+Rx+CyPx was significantly longer than that of non-treatment group. Although the number of cases was limited, additional metronomic chemotherapy was seemed to extend the survival time. The side effects of metronomic chemotherapy such as anorexia and vomiting were noted in some patients, but those were mild and controllable by usual medical supportive treatments.

In this study, the anti-tumor effect of metronomic chemotherapy using low-dose cyclophosphamide and piroxicam was investigated *in vitro* and *in vivo* experiments and clinical patients. Although thedefinitive mechanism of the anti-proliferative and anti-angiogenic activity was still unclear, metronomic cyclophosphamide and piroxicam was suggested to show the anti-tumor effects on canine oral MM cells and xenografted tissues. Moreover, clinical benefits in both extended survival periods and limited side effect were revealed in spontaneous canine oral MM cases. Further molecular experiments *in vitro* and *in vivo* and prospective case controlled study with large population of patients will be needed to clarify the effectiveness of the metronomic chemotherapy on canine MMs.