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General Introduction

Malignant melanoma (MM) is one of the most common oral malignancies in dogs, which accounts for 30 to 40% of all canine oral tumors. Biological behavior of the canine oral MM is highly malignant as with human MM (Smith *et al.*, 2002). It frequently invades surrounding tissues and its metastatic rate to lymph node, lung and other organs is reported in up to 80% (Liptak and Withrow, 2007). Although several treatments including surgery, radiation therapy and conventional chemotherapy have been attempted in canine oral MM patients (Ramos-Vara *et al.*, 2000), survival time in most dogs with oral MM is less than one year.

Recently, metronomic chemotherapy has been reported as a promising alternative treatment in canine cancer patients as with human patients (Borne *et al.*, 2010; Elmslie *et al.*, 2008). Metronomic chemotherapy generally refers to repetitive like a metronome, a musical instrument, and prolonged administration of low dose chemotherapy drugs with no prolonged drug-free breaks designed to minimize toxicity and target the endothelium or tumor stroma with the aim of tumor dormancy (Mutsaers, 2009). This is a marked contrast to the conventional chemotherapy or maximum-tolerated dose (MTD) chemotherapy for killing tumor cells but with significant side effects. The drugs being used for metronomic chemotherapy in human cases include cyclophosphamide and methotrexate that administered to cancer patients mainly by oral administration at short interval (Colleoni *et al.*, 2006; Colleoni *et al.*, 2002). Positive results were reported with several metronomic chemotherapy

regimens for human patients with various advanced or recurrent cancers, such as breast cancer (Colleoni *et al.*, 2002), multiple myeloma (de Weerdt *et al.*, 2001), ovarian cancer (Jurado *et al.*, 2008), malignant glioma (Reardon *et al.*, 2009) and glioblastoma (Kong *et al.*, 2006), and canine patients with soft tissue tumors (Elmslie *et al.*, 2008). Metronomic chemotherapy is favorable in cancer patients due to the low cost and the oral administration of the drugs that are key characteristics of this schedule and may offer important social advantages. In addition, this treatment schedule, showed the favorable effects in those patients by suppressing the progression of malignant disease and improving of quality of life (QOL) without high-grade toxicity (Marchetti *et al.*, 2012; Pasquier *et al.*, 2010).

While various mechanisms of the anti-tumor activity by the metronomic chemotherapy have been proposed, one of the major anti-tumor mechanisms of metronomic chemotherapy is supposed to be an anti-angiogenesis effect (Pasquier *et al.*, 2010) based on the endothelial cytotoxicity and the induction of anti-angiogenic protein, Thrombospondin-1 (TSP-1). Metronomic administration of chemotherapeutic agents showed both cytotoxic effects on intra-tumor endothelial cells and circulated endothelial progenitor cells (Bertolini *et al.*, 2003; Mutsaers, 2009). In addition, the metronomic chemotherapy showed the selectivity of endothelial cells, especially in tumor microenvironment without perturbation of normal susceptible tissue and normal physiological angiogenesis (Emmenegger *et al.*, 2004). Previous studies

revealed that some chemotherapeutic agents such as cyclophosphamide, methotrexate, vinblastine and paclitaxel, showed the 10 to 100,000 fold higher sensitivity to endothelial cells as compared with non-endothelial cells (Bocci *et al.*, 2002). In addition, metronomic administration of chemotherapeutic agents prevented the migration of endothelial progenitor cells during angiogenic process (Stoelting *et al.*, 2008).

It has been reported that chronic administration of cyclophosphamide at low dose (as a chemotherapeutic usage) induced an increase in expression of an endogenous anti-angiogenic protein, TSP-1 (Hamano *et al.*, 2004). TSP-1 exerts a potent inhibitory effect of angiogenesis by binding and displacing of pro-angiogenic factor, or vascular endothelial growth factor (VEGF) (Gupta *et al.*, 1999). An increase of TSP-1 in cancer patients by metronomic chemotherapy, balanced pro- and anti-angiogenic proteins that subsequently promoted vascular normalization of tumor vessels (Jain, 2005). To lose balance of angiogenic and anti-angiogenic proteins caused by accelerated production of a pro-angiogenic protein such as VEGF in tumor tissues turns on the angiogenic switch (Bergers and Benjamin, 2003). This angiogenic switch consequently induces an abnormal sprouting of tumor vessels characterized by a dilated-tortuous structure with less tight junction and poor pericyte coverage (Jain, 2005). These pathologic vessels cause the vascular leakage and tumor interstitial tissue hypertension that disturb the transportation of chemotherapeutic agents or

oxygen into tumor tissue. As a result, the pathologic vessels induce a resistance of tumor cells against chemotherapeutic agents or radiation therapy. Thus, the normalization of tumor vessels plays a key role for an effective cancer therapy (Carmeliet and Jain, 2011).

In contrast to several other tumors, a single use of metronomic cyclophosphamide in human MM revealed discrepant outcome; it caused a decrease in TSP-1 and a subsequent imbalance of angiogenic proteins and the low percentage of normal vessels in tumor tissues. However, this chemotherapeutic schedule decreased tumor microvessel density (MVD) in tumor tissues (Patten *et al.*, 2010). To obtain the optimal anti-angiogenic effect both in quantity and quality by metronomic cyclophosphamide in canine oral MM, the combination treatment with other pro-angiogenic inhibitors may be necessary to recall the balance of angiogenic proteins in tumor tissues (Jain, 2005).

As canine oral MM has been reported to show the high expressions of COX-2 and VEGF (Pires *et al.*, 2010; Taylor *et al.*, 2007), non-steroidal anti-inflammatory drugs (NSAIDs), which inhibit the cyclooxygenase-2 (COX-2) activity potently, are expected to be the beneficial additional drugs for metronomic chemotherapy in canine MM (Yoshida *et al.*, 2003). NSAIDs inhibit COX-2 activity, which converts arachidonic acid into prostaglandin (PGs) family. (Seibert *et al.*, 1994). Among those PGs, significant increase in PGE2 was observed in malignant tissues (Cha and

DuBois, 2007; Mohammed *et al.*, 2001). PGE2 signaling contributes to the tumor progression by regulating the tumor-associated angiogenesis via an induction of VEGF transcription (Ding *et al.*, 2005), inhibiting apoptosis and modulating tumor cell activities such as tumor cell proliferation, migration and invasion (Cha and DuBois, 2007). Therefore, NSAIDs may show the additive or synergistic effect on metronomic administration of cyclophosphamide at the low dose. Piroxicam is one of NSAIDs and has been used frequently in dogs with some kinds of tumors as the anti-tumor drug because of its inhibitory effect against COX-2 activity (Boria *et al.*, 2004). Recently the combination use of piroxicam and metronomic cyclophosphamide was revealed to show clinical benefits without severe side effect in canine incompletely resected soft tissue sarcoma (Elmslie *et al.*, 2008). Therefore, the combination of metronomic cyclophosphamide and piroxicam might be an effective protocol for the treatment of canine oral MM.

Based on these backgrounds, the objective of this study is to investigate the effectiveness of metronomic using low-dose cyclophosphamide and piroxicam in canine oral MMs by means of the anti-angiogenesis, anti-proliferation and induction of tumor cell apoptosis *in vitro* and *in vivo*. In Chapter 1, the anti-tumor effects of single and combination use of these drugs are evaluated in canine oral MM cell lines in the aspect of cell proliferation activities and related protein expressions. Chapter 2 is conducted to investigate the effects of single and combination use of these drugs on

tumor growth and angiogenic behaviors using a mouse xenografted model. Finally, clinicopathological features and the clinical effectiveness of the metronomic combination on the survival of canine oral MM patients are elucidated in Chapter 3.

Chapter 1

In vitro effect of metronomic cyclophosphamide and piroxicam on canine oral malignant melanoma cells

Introduction

Metronomic chemotherapy has recently been reported as a novel-promising treatment in cancer patients (Borne *et al.*, 2010; Elmslie *et al.*, 2008; Pasquier *et al.*, 2010). In this protocol, low dose chemotherapy drugs, such as cyclophosphamide, were administered to cancer patients mainly by oral administration at short interval. Among the several proposed mechanisms of its anti-tumor effect, an endothelial cytotoxicity and an anti-angiogenic effect are thought to play major roles (Pasquier *et al.*, 2010).

Tumor angiogenesis is known to be one of the important factors to promote the tumor progression (Hicklin and Ellis, 2005; Luong *et al.*, 2006) and to be correlated with the poor prognosis (Restucci *et al.*, 2003; Wolfesberger *et al.*, 2008). VEGF has been reported to be a crucial factor for tumor angiogenesis (Hicklin and Ellis, 2005), and several reports revealed the close correlation between VEGF induced tumor angiogenesis and poor prognostic outcome in cancer patients (Kato *et al.*, 2007; Taylor *et al.*, 2007). VEGF is a pro-angiogenic glycoprotein, which abundantly secreted by cancer cells (Murukesh *et al.*, 2010). During angiogenesis, VEGF interacts with its receptor and subsequently stimulates the proliferation and migration of intra-tumor endothelial cells (Nagy *et al.*, 2007). Metronomic chemotherapy using cyclophosphamide was reported to stimulate a production of endogenous anti-angiogenic protein TSP-1 from cancer cells, which inhibits the VEGF-modulate

angiogenic activity (Hamano *et al.*, 2004). TSP-1 modulates angiogenesis by binding and displacing VEGF (Gupta *et al.*, 1999). In addition, metronomic cyclophosphamide treatment was reported to recall the balance of pro- and anti-angiogenic proteins and subsequently normalize the phenotype of tumor vessels in several cancers (Jain, 2005), while discrepant results were obtained in human MMs (Patten *et al.*, 2010). Canine MM was supposed to share some common features with human MM (Smith *et al.*, 2002), but the effect of metronomic cyclophosphamide to pro- and anti- angiogenic factors was still unclear in canine MM.

In addition to the metronomic cyclophosphamide, various anti-angiogenic treatments targeting VEGF have been investigated (Albertsson *et al.*, 2006; Eichhorn *et al.*, 2004; Kamstock *et al.*, 2007; Lee *et al.*, 2009). NSAID is one of the promising candidate among them because of their inhibitory effect of cyclooxygenase (COX) (Yoshida *et al.*, 2003). PGE2, one of the major PGs induced by COX from arachidonic acid, is produced at the high level in tumor microenvironment and regulates several malignant signaling pathways including tumor angiogenesis by stimulation of VEGF transcription (Karamouzis and Papavassiliou, 2004). Therefore, inhibition of COX may induce anti-angiogenic effect.

Previous report revealed that intense COX-2 expression was more frequently detected in oral MM as compared with MM occurred in other areas (Pires *et al.*, 2010). Taylors and colleagues (2007) confirmed that dogs with oral MM showed high

VEGF expression in both of blood and tumor tissue and showed the correlation of expression level of VEGF to clinical stage and survival period (Taylor *et al.*, 2007). Therefore, combined use of NSAIDs, e.g. piroxicam, which used frequently in veterinary medicine, may be useful to inhibit tumor neovascularization through suppression of VEGF signaling (Zhang *et al.*, 2013) . In addition, NSAIDs may have other aspects of anti-tumor activities; the inhibition of the tumor cell proliferation (Verdina *et al.*, 2008), suppression of the tumor cell migration/invasion (Rozic *et al.*, 2001) and induction of the tumor cell apoptosis (Mohammed *et al.*, 2003) by affecting other signaling pathways such as ERK and Bcl-2.

The aim of this chapter was to evaluate the anti-tumor effect of each regimen of metronomic cyclophosphamide, piroxicam or their combination on canine MM cells *in vitro*. As a preliminary study, COX-2 and VEGF expressions were investigated in 6 canine MM cell lines to select one cell line and the treatment dose of each drug is determined for the following *in vitro* assays. To investigate the anti-tumor effect of each regimen, cell proliferative activities and anti-angiogenesis effects were evaluated by the expression of VEGF, TSP-1, and other related proteins such as PGE2, ERK, phosphorylated-ERK (p-ERK), and Bcl-2.

Materials and Methods

Cell culture

Six canine MM cell lines, CMeC-1, CMeC-2, KMeC, LMeC, CMM1 and CMM2 that established from melanoma patients in our laboratory (Inoue *et al.*, 2004; Ohashi *et al.*, 2001) (Table 1.1), were used in this study. Cell lines were maintained in RPMI-1640 medium (Wako Pure Chemical Industries Ltd., Osaka, Japan) supplemented with 10% heat inactivated fetal bovine serum (FBS) (Gibco BRL, NY, USA) and 5 mg/L gentamicin (Sigma-Aldrich, MO, USA) at 37 °C in humidified atmosphere with 5% CO_2.

Reagents and antibodies

All therapeutic reagents were purchased from Sigma-Aldrich Corporation. Cyclophosphamide monohydrate was dissolved in sterile distilled water at a concentration of 20 mg/mL. Piroxicam was dissolved in dimethyl sulfoxide (DMSO) at 50 mg/mL. Both stock solutions were kept under -20 °C until use.

The primary antibodies used for western blot analysis were as follows; monoclonal mouse anti-rat COX-2 antibody (dilution at 1: 1,000; BD Transduction Laboratories™, KY, USA), polyclonal rabbit anti-human VEGF antibody (1: 300; Santa Cruz Biotechnology, CA, USA), monoclonal mouse anti-human TSP-1 antibody (1: 500; Abcam, MA, USA), monoclonal rabbit anti-human ERK antibody

(1:1,000; Cell Signaling Technology, MA, USA), monoclonal rabbit anti-human p-ERK antibody (1:2,000; Cell Signaling Technology), monoclonal mouse anti-human Bcl-2 antibody (1: 1,500; BD Transduction Laboratories™) and monoclonal mouse anti-chicken actin antibody (1: 10,000; Millipore, CA, USA). To determine the proliferation index (PI), monoclonal mouse anti-human Ki-67 antibody (1: 100; Dako, Gostrup, Denmark) was used for immunocytochemistry.

Dose determination for *in vitro* treatment

Cell viability assay was performed to investigate the proper *in vitro* dose of each treatment drug using the cell proliferation kit I MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide, a yellow tetrazole) - based kit (Roche Diagnostics GmBH, Mannheim, Germany). Cells were seeded in quadruplicate in 96-well plates at a density 3 x 10^3 cells per well in FBS containing RPMI medium. After 24 hours of initial culture, the culture medium was exchanged to the treatment medium containing cyclophosphamide or piroxicam and incubated at 37 °C with 5% of CO_2 for another 48 hours. The concentration of cyclophosphamide was 0.0001, 0.001, 0.01, 0.1, 1 and 10 mg/mL and the concentration of piroxicam was 0.01, 0.05, 0.1, 0.25, 0.5, 0.75 and 1 mg/mL. After the incubation, the cell proliferation kit I MTT was applied. Cell viability was measured by the absorbance between 550 – 690 nm assessed with microplate ELISA reader (Bio-Rad Laboratories, Hertfordshire,

UK). A treatment medium without cells was used as the negative control, while media with cell growth under no treatment was represented as a positive control with 100% cell viability.

The treatment dose of low-dose cyclophosphamide was determined from the highest non-toxic concentration, and the dose of piroxicam was the fifty percentage of inhibitory concentration (IC50) that determined through linear regression equation.

Cell morphology and proliferation

5 x 10^4 cells were seeded in 12-well culture plates and incubated for 24 hours. Subsequently, culture medium were replaced by each treatment medium; no drug (control), low-dose cyclophosphamide (CyLD), piroxicam (Px) and the combination treatment (CyPx). After treatment for 0, 3, 6, 12, 24 and 48 hours at 37 °C with 5% of CO_2, morphological features of cells were observed under light microscopy.

Cells that treated with each regimen were collected and counted using trypan blue exclusion assay at each time point.

Cell viability

Cell viability after each treatment at 48 hours was investigated by a cell proliferation kit, I MTT in accordance with previous information. All treatment groups were examined in quadruplicate.

Immunocytochemistry for PI

Cells at a density of 5 x 10^3/ well were seeded onto Lab II 8-well chamber slides (Thermo Fisher Scientific, IL, USA). After 24 hours of incubation, the medium were exchanged to a new medium with or without each treatment reagent and incubated at 37 °C with 5% of CO_2 for another 48 hours. After that, the culture medium was aspirated and cells were fixed with ice-cold methanol at -10 °C for 5 minutes and air-dried. After rehydration with phosphate buffer saline (PBS) for 3 times, cells were treated with 0.3% hydrogen peroxide in distilled water at room temperature for 10 minutes to inactivate the endogenous peroxidase. Nonspecific protein binding was saturated by 5% normal goat serum (Sigma-Aldrich) in PBS with 0.1% Tween-20 (PBS-T) at room temperature for 1 hour, and then cells were incubated with a primary antibody against Ki-67 at 4°C overnight. After washing with PBS-T 3 times, cells were incubated with a labeled polymer solution containing the horseradish peroxidase (HRP)-conjugated antibodies against mouse immunoglobulin (EnVision™-HRP labeled polymer, DAKO) at room temperature for 30 minutes before visualizing with liquid 3,3'-diaminobenzidine (DAB)/hydrogen peroxidase solution (DAKO) and counterstained with Mayer's hematoxylin. The PI was calculated as the percentage of Ki-67 positive stained cell among 1,000 cells.

PGE2 production

Treatment media at 12, 24 and 48 hours were collected and kept at -80°C. PGE2 concentration in the medium was detected by a Prostaglandin E2 EIA kit (Cayman, MI, USA) in accordance with the manufactured direction. PGE2 concentrations were determined using microplate ELISA reader (Bio-Rad Laboratories) at a wavelength between 405 and 420 nm. PGE2 concentrations of each treatment were normalized by the number of tumor cells at each time point and expressed as the concentration of PGE2/10^4 cells.

Western blot analysis

Sub-confluent canine MM cells were washed 3 times in ice-cold PBS before lysing in RIPA buffer (50 mM Tris-HCl, 150 nM NaCl, 5 nM EDTA, 1% Triton-X, 0.1% SDS, 4 mM Pefabloc SC (Roche Diagnostics, Basel, Switzerland), 5 μg/mL aprotinin, 5 μg/mL leupeptin, 10 mM NaF, 2 mM Na_3VO_4 and 1 complete mini tablet (Roche Diagnostic)). Lysates were spin at 15,000 rpm at 4 °C for 20 minutes, subsequently supernatant was collected. Protein concentration was measured by a spectrophotometer using BCA protein assay reagent (Thermo Fisher Scientific). After adding 2x sample loading buffer and denaturing at 98 °C for 5 minutes, equal amount of protein were resolved on sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto polyvinylidine fluoride (PVDF)

membranes (Bio-Rad Laboratories, CA, USA). The membrane was blocked with 5% non-fat dry milk in Tris Buffer Saline with 0.1% of Tween-20 (TBS-T) at room temperature for 1 hour. The blots were incubated with primary antibodies at 4°C overnight with vigorous agitation. After washing with TBS-T, membranes were incubated with HRP-conjugated antibody against mouse IgG or rabbit IgG (GE healthcare, NJ, USA) at room temperature for 1 hour with vigorous agitation. Then, the antigen-antibody complexes of protein were visualized by using the enhanced chemiluminescence (ECL) plus detection system (GE healthcare).

Statistical analysis

Statistical analyses were performed using Prism5.0 (Graphpad Software, CA, USA). Data were expressed as the mean with standard errors (SEM). Linear regression was used for IC50 determination of piroxicam. The differences between treatment groups were analyzed by ANOVA, followed by post-hoc analysis using Tukey's LSD methods. The cell numbers and PGE2 concentrations of each regimen at each time point were statically tested by 2way ANOVA followed by post-hock analysis using Bonferroni posttest. Differences were statistically significant when $P < 0.05$.

Results

COX-2 and VEGF protein expressions in canine MM cell lines

Prior to the dose determination, expressions of COX-2 and VEGF in 6 canine MM cell lines were evaluated by western blot analysis (Fig. 1.1). Among these cell lines, CMM1 cells showed highest expression of COX-2, while its expression was lowest in LMeC cells. For pro-angiogenic protein, all cell lines revealed similar high expression of VEGF. According to these results, CMM1 cell line was selected for the following experiments (Fig. 1. 2).

Cell viability and treatment dose determination on CMM1 cells

After incubation with each concentration of cyclophosphamide or piroxicam, viability of CMM1 cells was evaluated by the cell proliferation kit I MTT. The highest non-toxic concentration of cyclophosphamide was 0.1 mg/mL, and the IC50 dose of piroxicam, which calculated by linear regression equation, was 0.38 mg/mL (Fig. 1. 3). These treatment doses were used in all following *in vitro* studies.

Cell numbers and morphological appearance of CMM1 cells after treatments

Fig. 1.4 and Fig. 1.5 showed the cell numbers and morphological appearance of CMM1 cells after treatment with each regimen; control, CyLD, Px and CyPx group, respectively. The cell numbers in each group were similar until 12 hours. At 24 hours,

the cell numbers of Px and CyPx groups were significantly lower than that of the CyLD group ($P < 0.001$ and $P < 0.05$ for Px and CyPx groups, respectively). At 48 hours, the cell numbers of regimens that used piroxicam (Px and CyPx group) were significantly lower than those of CyLD and control group ($P < 0.001$) (Fig. 1.4).

Morphological appearances of CMM1 cells treated with each regimen were similar during 0 – 12 hours in accordance with the cell number result. However, at 24 and 48 hours, the morphological appearance of Px and CyPx groups also changed to be less spindle shape and the cell number of these groups was lower than that of other groups with a large amount of dead floating cells in the treatment medium (Fig. 1.5).

Cell viability of CMM1 cells after treatments

Using cell proliferation kit I MTT, viable cells after 48 hours of treatments were measured. Regimens that use piroxicam (Px and CyPx group) significantly lowered metabolic cells compared to those of control or CyLD group ($P < 0.001$). Besides, metabolic cell of CyPx group was significantly lowered than that of Px group ($P < 0.05$; Fig. 1.6)

PI of CMM1 cells after treatments

Fig. 1.7 showed immunocytochemical findings of CMM1 cells after staining for Ki-67 (A) and the result of PI of CMM1 cells after treatments with each regimen

(B). After treatment at 48 hours, PI of Px and CyPx groups were similar and were significantly lower than those of control and CyLD groups ($P < 0.001$).

PGE2 concentration in treatment medium

Fig. 1.8 showed PGE2 concentrations in treatment medium at 12, 24 and 48 hours. The PGE2 concentration in regimens that used piroxicam (Px and CyPx groups) was significantly lower than other groups at all time points ($P < 0.01$ at 12 hours and $P < 0.001$ at 24 and 48 hours). At 12 hours, the concentration of the CyLD group was significantly higher than that of the Px group ($P < 0.05$). The CyLD group revealed significantly suppressed PGE2 production comparing to that of the control group ($P < 0.05$) at 24 hours, but no difference at 12 and 48 hours.

Protein expressions in CMM1 cells after treatments

Fig. 1.9 showed VEGF, TSP-1, ERK, p-ERK and Bcl-2 protein expressions in CMM1 cells after treatment with CyLD, Px or CyPx at 0 – 24 hours. For angiogenic protein expressions, remarkable change of VEGF expression was not noted in all treatment groups. On the other hands, the Px group showed slight increase of TSP-1 at 6 and 12 hours, while the expression of TSP-1 was chronologically decreased in CyLD and CyPx groups. Regarding to ERK/p-ERK, which relate to the cell proliferation, the expression of ERK was similar in all treatments. In contrast, the p-

ERK expressions of all treatments were gradually decreased after the 3 hours, especially in the Px group. The expression of anti-apoptotic protein, Bcl-2 decreased in Px and CyPx groups since 3 hours, but showed no change in the CyLD group at all time points.

Discussion

In the preliminary study, COX-2 and VEGF expressions in canine MM cell lines were evaluated to select one to use in the following experiments. Among 6 MM cell lines established in my laboratory, CMM1 cells revealed the highest expression of COX-2 protein. Although the number of cell lines tested in this study was limited, relatively high COX-2 expressions were observed in cell lines established from the tumor resected from the oral cavity. This finding may reflect the previous report that MM of oral area showed high intense and percentage of COX-2 expression than those of other areas (Pires *et al.*, 2010). In contrast to the COX-2 expression, all cell lines showed almost similar level of VEGF expression. The expression of VEGF was also reported to be regulated by other intrinsic factors, such as hypoxia inducible factor 1 (HIF-1) (Ahluwalia and Tarnawski, 2012; Minet *et al.*, 2000). Inconsistent expressions of COX-2 and VEGF in these canine MM cell lines might suggest that the VEGF expression was regulated not only by COX-2 stimulation, but also by other intrinsic factors.

To determine the proper dose of each drug, cell viability of the selected CMM1 cells treated with cyclophosphamide and piroxicam was evaluated by MTT assay. Although several metronomic chemotherapeutic protocols were designed in experimental and clinical study of human and canine tumors, the proper combination and dose of each drug have been still unknown. In this study, the highest non-toxic

dose of cyclophosphamide and the IC50 dose of piroxicam were determined for the following experiment that stimulates the metronomic chemotherapy.

According to the results of the cell numbers, the metabolic and proliferation activities, the anti-tumor effects were thought to be mainly caused by COX-2 inhibition by piroxicam, which induced the decrease of PGE2, Bcl-2 and p-ERK levels. Additionally, combination regimen with cyclophosphamide revealed superior effects than single use of piroxicam on the metabolic activity and the production of PGE2, while this regimen did not show additional effect on tumor cell number and the proliferation index. The discordant results by this regimen might be caused by the inhibition of some tumor cell activities, e.g. mitochondrial activity. Further study of the effect of combination treatment on the molecular level of tumor in *in vitro* should be warrant.

Single use of low dose cyclophosphamide revealed some effects on PGE2 production and p-ERK expression. At 12 - 24 hours, significantly lower production of PGE2 was detected by single use of low dose cyclophosphamide as compared with control. With lower PGE2 production after *in vitro* treatment with metronomic cyclophosphamide as observed in this study, some PGE2 related signaling in the cell activity may be suppressed (Cha and DuBois, 2007), e.g. the decrease of p-ERK expression seen in this study. Further molecular studies of dose-and time-schedule

dependence in *in vitro* metronomic cyclophosphamide on PGE2 production are necessary.

In this study, VEGF expression did not show any differences among regimen even by piroxicam treatment. As the inhibition of PGE2 production and suppression of p-ERK and Bcl-2 expressions were observed in Px and CyPx groups, piroxicam was supposed to inhibit the VEGF expression to some extent. No difference in VEGF expressions among treatment groups might suggest that other factors regulated the regulation of VEGF expression dominantly in CMM1 cells. In contrast, TSP-1, the endogenous anti-angiogenic protein produced by tumor cells, was increased with the single use of piroxicam, while the opposite results in low dose cyclophosphamide and combination regimen. Anti-angiogenic activities of these treatment regimens were not clear in culture conditions of this *in vitro* experiment. To clarify them, *in vivo* experiments were thought to be needed in the aspect of not only effects on tumor cells but also tumor microenvironment.

In conclusion of this *in vitro* study, a single use of piroxicam and a combination of low dose cyclophosphamide and piroxicam revealed a potent anti-tumor effect on canine oral MM cells owing to the decrease of cell number, cell metabolic activity and proliferative activity. The decrease of p-ERK and Bcl-2 following the reduction of PGE2 production might induce these anti-tumor effects.

For anti-angiogenic properties, piroxicam showed the induction of anti-angiogenic protein, TSP-1, but entire regimens did not show the effect on the expression of pro-angiogenic factor, VEGF. This *in vitro* study suggested that these regimens were effective in canine oral MM in the aspect of anti-proliferative activity; however the anti-angiogenic effect was controversial. Therefore, further *in vivo* study will be needed to confirm the anti-tumor effect of this regimen and clarify the anti-angiogenic activities on tumor vessels.

Table 1.1 Canine MM cell lines derived from spontaneous patients.

Cell line name	Origin	Breed	Age (years)	Primary site	TNM classification	Clinical stage
CMeC-1	Primary tumor	Chow chow	11	Skin	T3N1M0	IV
CMeC-2	Lung metastasis of CMeC-1 xenografted mice					
KMeC	Primary tumor	Mongrel	14	Oral	T3N1M0	IV
LMeC	Metastatic lymph node	Beagle	9	Oral	T4N1M0	IV
CMM1	Primary tumor	Toy poodle	12	Oral	T2N1M0	III
CMM2	Primary tumor	Mongrel	13	Oral	T2N0M0	II

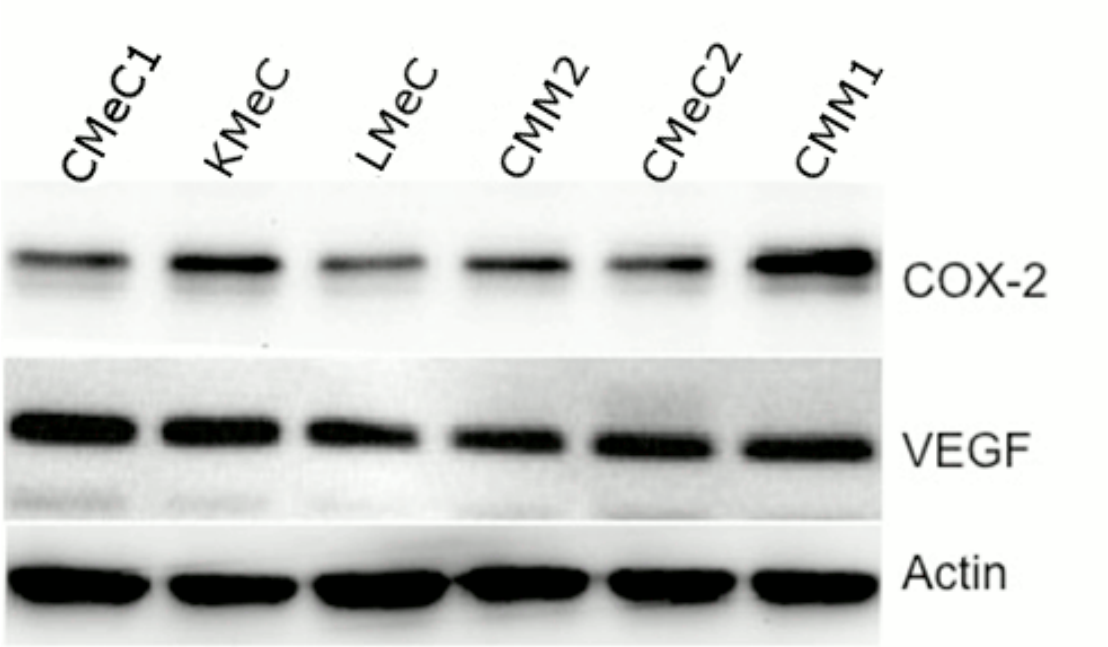


Fig. 1.1 Protein expressions of COX-2 and VEGF in canine MM cell lines by western blot analysis. CMM1 cells showed the highest expression of COX-2, while all cell lines showed the similar expression of VEGF.

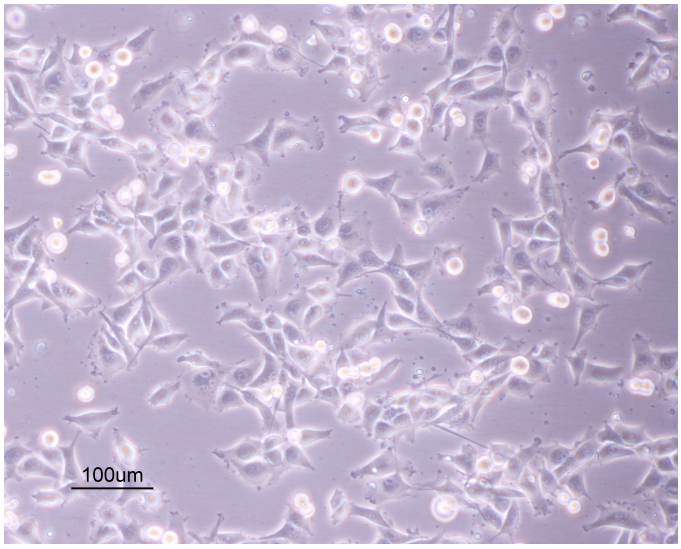


Fig. 1.2 Morphological appearance of CMM1 cells.

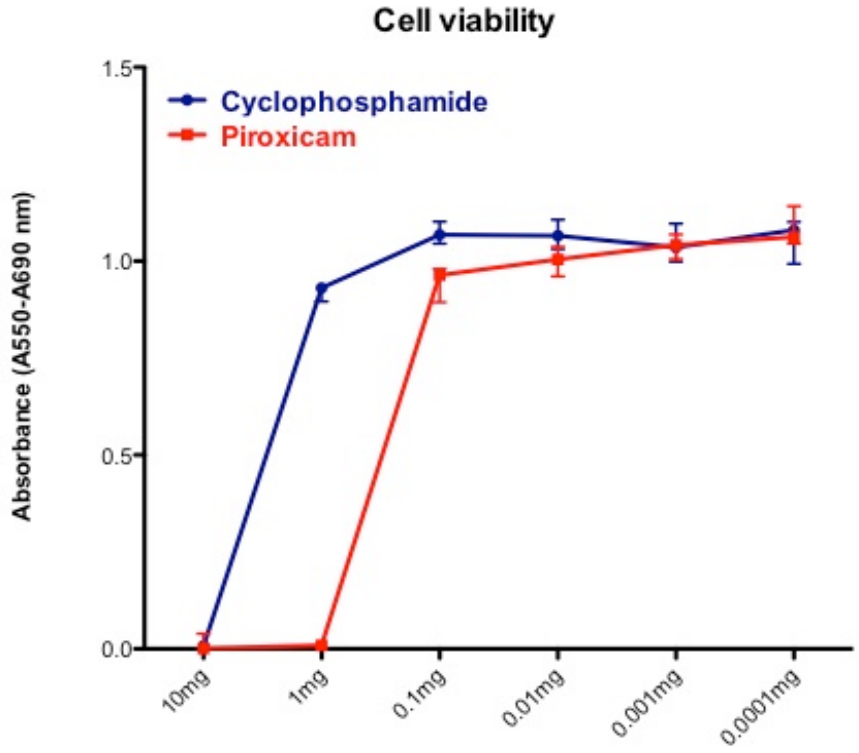


Fig. 1.3 Cell viability of CMM1 cells after treatment with cyclophosphamide or piroxicam for 48 hours evaluated by the cell proliferation kit I MTT. The highest non-toxic concentration of cyclophosphamide was 0.1 mg/mL and the IC 50 dose of piroxicam was 0.38 mg/mL.

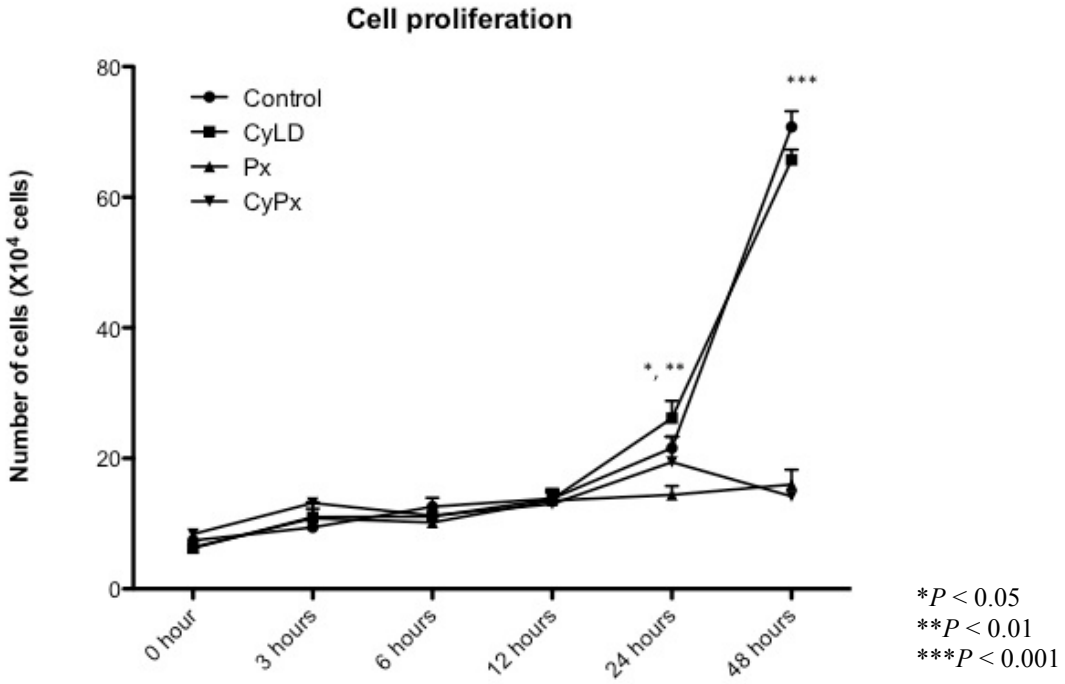


Fig. 1.4 CMM1 cell number of each treatment groups at 0 – 48 hours detected by trypan blue exclusion assay. Cell numbers of each group were low and comparable during 0 – 12 hours. However, at 24 hour, CMM1 cell numbers were slightly different in treatment groups that used piroxicam (Px and CyPx) and did not use piroxicam (control and CyLD). Significant difference in viable cell number was noted between the control and Px groups ($P < 0.05$), between the CyPx and CyLD groups ($P < 0.05$) and between the CyLD and Px groups ($P < 0.01$). At 48 hours, treatment that used piroxicam (Px and CyPx) significantly decreased cell number than those of control and CyLD group ($P < 0.001$).

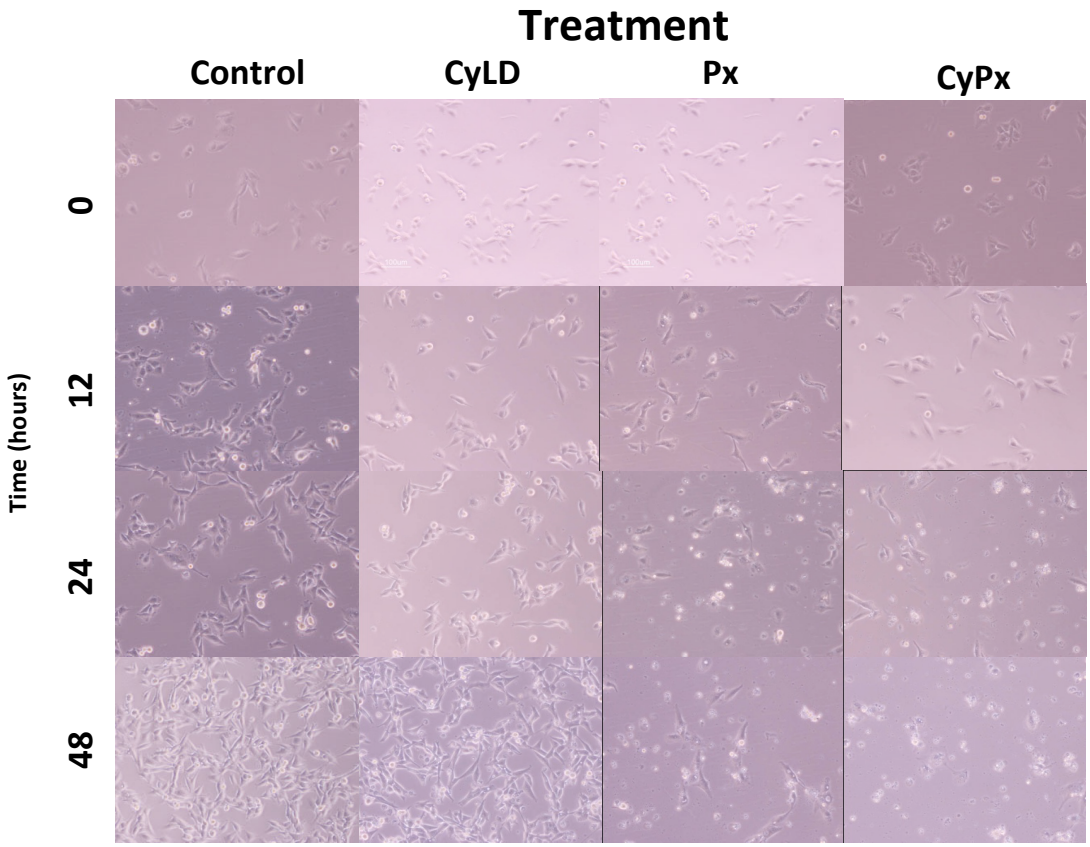


Fig. 1.5 Morphological appearances of CMM1 cells of each treatment at 0 – 48 hours. Cell number and morphological appearance were similar during 0 – 12 hours. However, since 24 hours, CMM1 cell numbers were significantly lower with larger amount of dead floating cells and morphological appearances were less spindle shape in treatment groups that used piroxicam (Px and CyPx) compared to those of control and CyLD group, especially at 48 hours ($P < 0.001$).

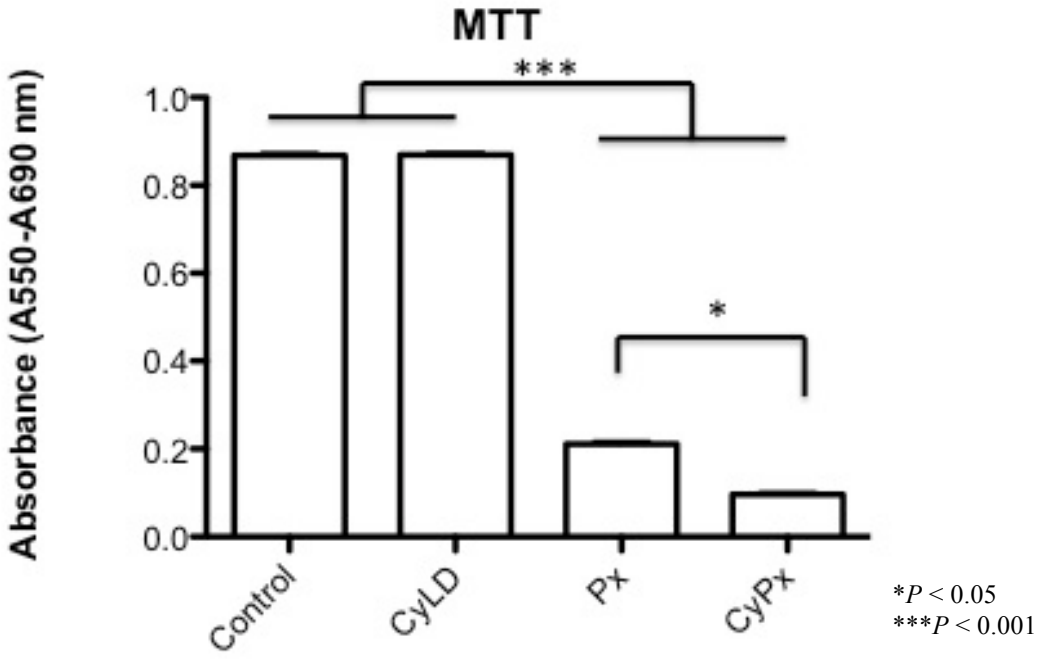
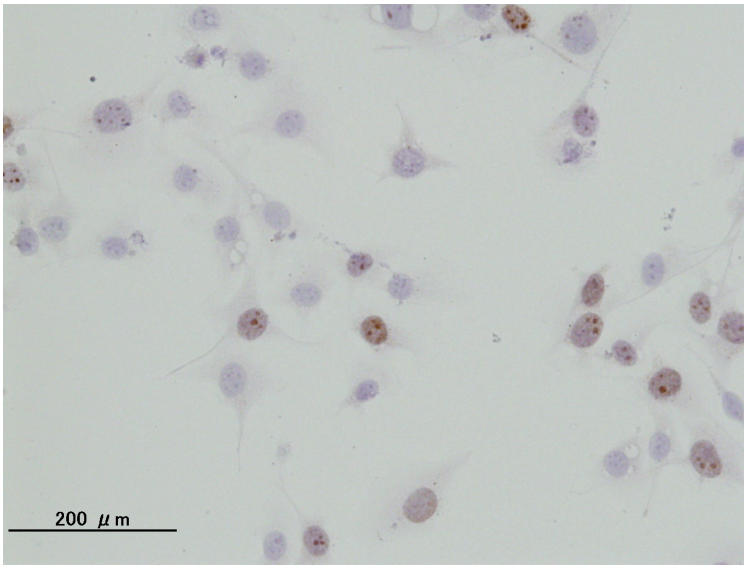
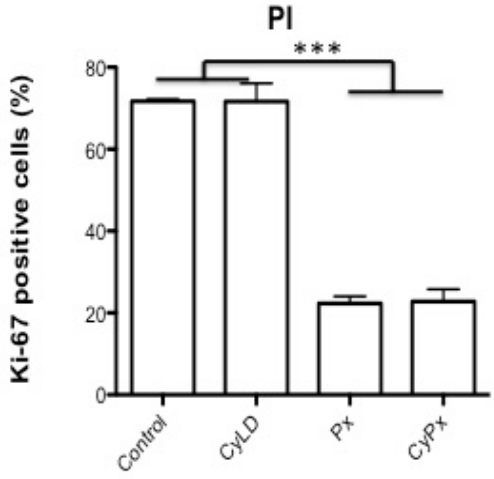


Fig. 1.6 Cell viability of CMM1 cells after treatment with each regimen evaluated by cell proliferation kit I MTT. Treatment that used piroxicam (Px and CyPx groups) significantly lowered the metabolic CMM1 cells compared to those of control and CyLD group ($P < 0.001$). Beside, CyPx group revealed significantly lower metabolic cells than those of Px group ($P < 0.05$).

A.





B.

***$P < 0.001$

Fig. 1.7 Ki-67 immunocytochemistry (A) and result of proliferation index (PI) (B) of CMM1 cells after treatments with each treatment. PI was comparable between control and CyLD groups, and between Px and CyPx groups. Treatments that used piroxicam (Px and CyPx groups) significantly lowered the PI than those of control and CyLD groups ($P < 0.001$).

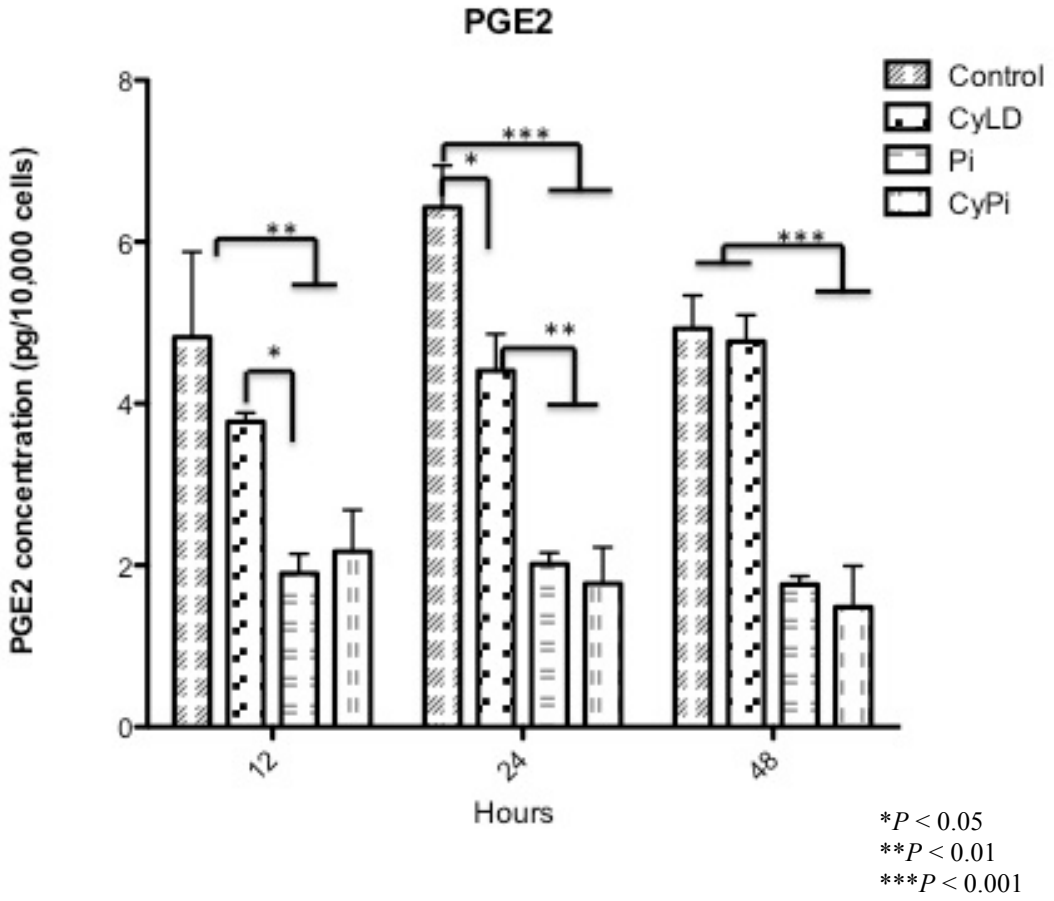


Fig. 1.8 Prostaglandin E2 (PGE2) concentrations in treatment medium of each treatment at 12, 24 48 hours. Treatments that used piroxicam (Px and CyPx groups) showed significantly lower PGE2 concentration than those of control and CyLD groups ($P < 0.01$ at 12 hours, and $P < 0.001$ at 24 and 48 hours). CyLD group revealed potent effect to inhibit PGE2 production than that of control only at 24 hours ($P < 0.05$).

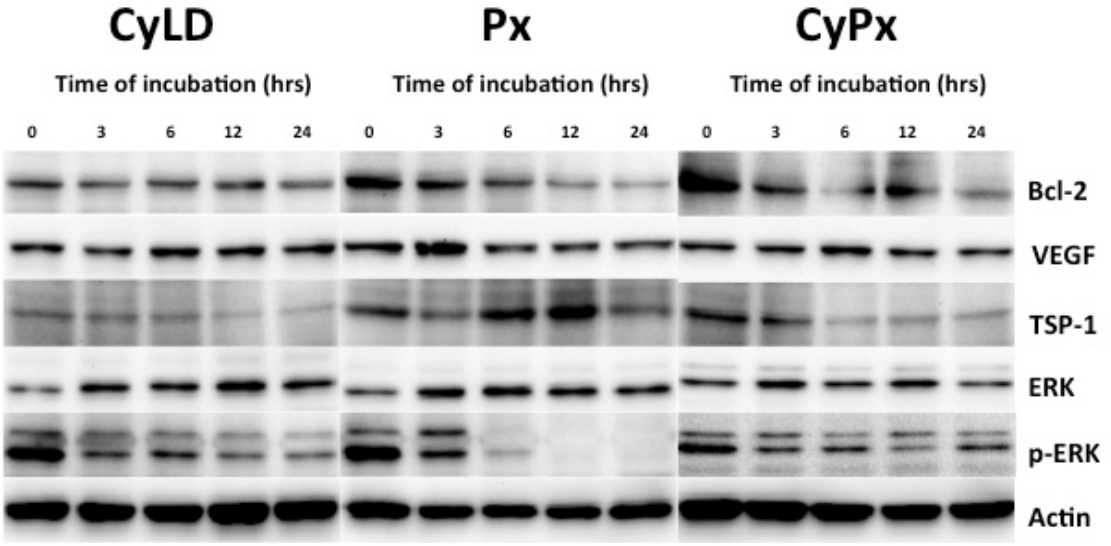


Fig. 1.9 Protein expressions of CMM1 cells after treatment with each regimen from 0 – 24 hours. Px and CyPx groups showed the decrease of Bcl-2 since 3 hours of treatment. All regimens did not effect on VEGF expression. Endogenous anti-angiogenic TSP-1 was slightly decreased in CyLD, slightly increased in Px and gradually decreased in CyPx. Although, all regimens did not significantly change in ERK, p-ERK in all treatments was decreased since 3 hours, especially in Px group.

Chapter 2

Effect of metronomic cyclophosphamide and piroxicam in a canine oral malignant melanoma-xenografted mouse model

Introduction

Neovascularization is a key requirement for tumor growth and metastasis as it provides nutrients and oxygen for the highly metabolism of tumor tissue (Hicklin and Ellis, 2005). Typical tumor vessels are considered to be pathogenic because of poor mural cell coverage, which is caused by high levels of pro-angiogenic protein, VEGF in the tumor tissues (Jain, 2005). These pathogenic vessels induce the tumor interstitial hypertension and poor oxygenation, which in turn cause the tumor resistance against the chemotherapy and radiotherapy (Carmeliet and Jain, 2011). Several studies have shown that high expressions of tumor vascular markers such as VEGF and micro vessel density (MVD) were associated with a poor outcome in canine solid malignant tumors (Luong *et al.*, 2006; Restucci *et al.*, 2003; Taylor *et al.*, 2007; Wolfesberger *et al.*, 2008). Based on these findings, anti-angiogenic strategies focused on both quantity and quality is needed for effective inhibition of tumor neovascularization.

Among several anti-angiogenic treatments, metronomic chemotherapy is a relatively recent addition. One of the most important mechanism of metronomic chemotherapy is supposed as the inhibition of tumor neovascularization through endothelial cytotoxicity (Mutsaers, 2009) and another is the normalization of tumor vessels by increasing of the endogenous anti-angiogenic protein, TSP-1 (Hamano *et al.*, 2004). Metronomic administration of chemotherapeutic agent, such as

cyclophosphamide, was reported to increase the level of TSP-1 and normalize pathogenic vessels by balancing pro- and anti-angiogenic proteins in human cancer patients (Jain, 2005). However, in human MM, metronomic cyclophosphamide was reported to cause an imbalance of angiogenic proteins by reducing the level of TSP-1, which resulted in increased number of pathogenic vessels in tumor tissues, although this treatment reduced the MVD (Patten *et al.*, 2010). Canine MM is supposed to share some common features with human MM (Smith *et al.*, 2002). Indeed, the TSP-1 expression of canine MM cells was decreased after treatment of low-dose cyclophosphamide in the previous chapter.

As the combination drug with metronomic cyclophosphamide, piroxicam was supposed to be a good candidate, because the high expression of COX-2 has been detected in canine oral MM tissue (Pires *et al.*, 2010) and in 6 canine MM cell lines, which was evaluated in Chapter 1. In CMM1 cells, which showed high COX-2 expression, significant reduction of cell proliferation, cell viability and PI accompanied with low PGE2 production were observed after treatment of piroxicam. Changes in related proteins, p-ERK and Bcl-2, might also affect these anti-tumor effects. In addition, the combination of metronomic cyclophosphamide and piroxicam showed more potent effect in cell viability than other regimens. However, this *in vitro* study had some limitations because of the difference from *in vivo* microenvironment such as lack of blood and lymphatic vessels, immune cells, or other stromal.

The purpose of this chapter was to investigate the anti-tumor effect of metronomic cyclophosphamide, piroxicam and their combination in a canine oral MM cell xenografted mouse model. To establish the suitable model for simulating the tumor microenvironment and to evaluating the effect of metronomic chemotherapy *in vivo*, the time course of tumor progression in the aspects of cell proliferation and angiogenesis in the CMM1 cell transplanted mouse model was evaluated in Section 1. In the following section (Section 2), the anti-tumor effect of each regimen was investigated in the aspects of tumor growth, angiogenesis and apoptosis.

Section 1: Tumorigenesis of a canine oral MM-xenografted mouse model

Materials and Methods

Cell culture

According to the results of Chapter 1, CMM1 cell line was used for an *in vivo* xenografted mouse model. CMM1 cells were maintained in the same condition as described in Chapter 1.

Primary antibodies

Primary antibodies used for immunohistochemistry and western blot analysis, were against COX-2, VEGF, ERK, p-ERK and Ki-67, which were purchased as described in Chapter 1. In addition, polyclonal rabbit anti-human CD31 antibody (Abcam) was used for immunohistochemical staining to detect the endothelial cells. Information of each antibody dilution in both for immunohistochemical staining and western blot analysis was shown in Table 2.1.1.

Animals

Five-week-old female BALB/c nude mice (SLC, Tokyo, Japan) were used as recipients of CMM1 cell xenotransplantation. The mice were maintained under specific pathogen-free condition under 24 ± 1°C, 40-70% of humidity and 12 hours of

light and dark cycle. Mice were given sterilized food (CL-2; Clea Japan, Tokyo, Japan) and water *ad libitum*.

Xenotransplantation and evaluation of primary tumor growth

This experiment was approved by the Animal Care Committee of the Graduate School of Agricultural and Life Sciences, the University of Tokyo. To evaluate tumor cell behaviors such as tumor growth, angiogenesis and apoptosis in a nude mice xenografted model, 1.5 x 10^7 CMM1 cells, which suspended in 200 μL of serum-free medium, were subcutaneously injected into their back. After transplantation, tumor size was measured 3 times a week by a caliper and its volume was calculated using the following formula:

$$\text{Tumor volume } (mm^3) = (\text{length} \times \text{width}^2)/2$$

After the transplantation, 6 mice, which were randomly selected, were sacrificed every 10 days and primary tumor tissues were collected tissues at each time point. The maximum time point was decided based on the tumor-related clinical signs and body weight loss. The collected tissue at each time points was divided for histopathology and western blot analysis.

Histopathology and immunohistochemical staining

After sacrifice, primary masses were fixed in 10% neutral buffered formalin for 24 hours prior to tissue processing for histopathological investigation. A series of 4 μm-thick, paraffin sections were stained with hematoxylin-eosin or immunostained for Ki-67, endothelial cell, or VEGF. Immunohistochemical staining was done using DAKO ENVISION+ kit/HRP (DAKO diagnostic Japan, Kyoto, Japan). Sections were deparaffinized in xylene, subsequently rehydrated through graded ethanol, followed by distilled water. To retrieve protein antigens on section tissues, sections were incubated in 10mM sodium citrate buffer, pH 6.0, at 121°C for 10 minutes. After cooling down for 30 minutes, the endogenous peroxidase on sections were inactivated by the incubation with 0.3% hydrogen peroxidase at room temperature for 10 minutes, subsequently washing by PBS-T. Nonspecific protein binding was saturated using 5% normal goat serum (Sigma-Aldrich) in PBS-T at room temperature for 1 hour. All sections were incubated with primary antibodies at 4°C overnight. After washing with PBS-T, sections were labeled with a polymer solution containing HRP-conjugated antibodies against mouse or rabbit immunoglobulin (EnVision^{TM}-HRP labeled polymer, DAKO) for 30 minutes, and tissue sections were visualized with a liquid DAB/hydrogen peroxidase solution (DAKO). Nuclear counter staining of each section was done by Mayer's hematoxylin before dehydration by graded ethanol, clearing in xylene and covering with a cover glass slide. Appropriate positive controls

were used to assess the specificity of the reactions, while negative controls were included where the primary antibody was replaced by PBS-T.

Evaluation of PI, MVD and VEGF expression

To evaluate PI for each sample, 5 high-power fields (400x) were randomly selected. Ki-67-positive cells were counted and its percentage among 1,000 visible tumor cells was calculated as PI.

For MVD expression of each section, 5-hot spot areas of CD31-positive area (200x) were selected, and CD31-positive vessels were counted. The number of CD31-positive vessels/mm^2 was defined as the MVD expression for each sample.

The expression of VEGF staining of each sample were evaluated from 5 randomly high power fields (400x) and scored by the method that was previously reported in the canine oral MM tissue (Taylor *et al.*, 2007). Staining intensity was recorded as the following grades; (0) none, (1) weak, (2) moderate and (3) strong.

Western blot analysis

The primary tumor tissues collected from nude mice were immediately lysed in the CelLytic^{TM} MT mammalian tissue lysis/extraction reagent (Sigma-Aldrich) with complete mini tablet (Roche Diagnostic). The immunoblotting analysis was

performed as described in Chapter 1 for investigating the protein expressions including COX-2, VEGF, ERK, p-ERK and actin.

Statistical analysis

Statistical analyses were performed using Prism5 (Graphpad Software). Data were expressed as the mean and SEM. The differences among groups were analyzed by one-way ANOVA, followed by post-hoc analysis using Tukey's LSD methods. Size of tumors at different time points among different groups were statically tested by 2 way ANOVA followed by post-hoc analysis using Bonferroni posttest. All differences with a P-value < 0.05 were defined as statically significant.

Results

Tumor growth in CMM1 cell xenografted mice

After transplantation of CMM1 cells, primary masses rapidly developed in xenografted mice (234.8 ± 57.3, 900.1 ± 208.6, 1999.2 ± 573.3 and 2815.8 ± 549.7 mm^3 in 10, 20, 30 and 40 days after transplantation, respectively) (Fig. 2.1.1). Ulcer on the surface and necrosis in the center of primary masses were found after three or more weeks of transplantation (Figs. 2.1.1A and 2.1.2). At 40 days after transplantation, the body condition of xenografted mice became poor and the body weight of these xenografted mice was significantly reduced as compared with those at 20 and 30 days ($P < 0.01$) (Figs. 2.1.1 and 2.1.3).

Fig. 2.1.4 showed histopathological findings of primary mass of CMM1 cell-xenografted mice. Histopathology of primary tumor mass was compatible to the feature of sarcoma. Fewer intra-tumoral neovascularization was observed at 10 days after transplantation, while more number of vessels has been detected after 20 and more days of transplantation.

PI, MVD and VEGF expression in CMM1 xenografted primary mass

Fig. 2.1.5A shows the result of immunohistochemical staining for Ki-67 on tumor tissues in CMM1-xenografted nude mice. PI of the tumor tissue at 10 days was

significantly higher than that of 20 days ($P < 0.05$), but there were no significant differences between PI of 10 days and those of other time points. (Fig. 2.1.5B)

The MVD of primary tumor tissues in CMM1 cell-xenografted nude mice is shown in Fig. 2.1.6A. MVD was progressively increased during 10 to 20 days and 20 to 30 days after transplantation ($P < 0.001$), and then reached a plateau during 30 and 40 days after transplantation (Fig. 2.1.6B).

The result of the VEGF expression by immunohistochemical staining is shown in Fig. 2.1.7. The VEGF expression showed two distribution pattern; diffuse and granular cytoplasmic staining (A). VEGF score were medium-high level in all time points and there were no significant difference between each time point (Fig. 2.1.7B)

Protein expressions in CMM1 xenografted primary mass

Fig. 2.1.8 shows the protein expressions in tissues developed in CMM1-xenografted nude mice tumor investigated by western blot analysis. At 10 days after transplantation, COX-2 showed high expression. However, its expression revealed less intensity during 20, 30 and 40 days. Pro-angiogenic protein, VEGF showed moderate expression during 10 to 30 days after transplantation. However, at 40 days after transplantation, the VEGF expression was more intensive than that of other time points. ERK and p-ERK were used to investigate tumor cell activity. ERK of all time

points revealed medium to high expressions, however, p-ERK revealed high expressions during 20 to 30 days after transplantation.

Discussion

Based on the result of *in vitro* investigations, CMM1 cell line was used for a xenografted mouse model. This cell line was established from a canine oral MM patient with stage III (T2bN1M0) (Ohashi *et al.*, 2001). After transplantation, tumor masses grew rapidly in nude mice. PI was highest in 10 days after transplantation, and then lowered. The cause of the low PI after 20 days of transplantation may have been due to an insufficient tumor microenvironment to provide enough nutrition and oxygen to tumor cells. This result coincided with the changes in MVD that significantly increased from 10 days to 20 days. In the 40 days group, MVD was similar level to that of 30 day, though the tumor mass still grew in size. Low MVD on day 40 may cause the lack in nutrition and oxygen in the central area of the mass resulting in the necrosis, which was observed three or more weeks after transplantation. In contrast to MVD, the VEGF expression on tumor tissues by immunohistochemical staining did not change during the experimental period. Inconsistency of MVD and VEGF expression might be caused by the limitation of recruiting endothelial cells for neovascularization.

The expression of COX-2 protein in the primary mass in a CMM1 cell xenografted nude mice model was highly detected only at the 10 days after transplantation. The lower COX-2 in the following period may have been caused by unsuitable microenvironment because of ectopic or xenotransplantation. In addition,

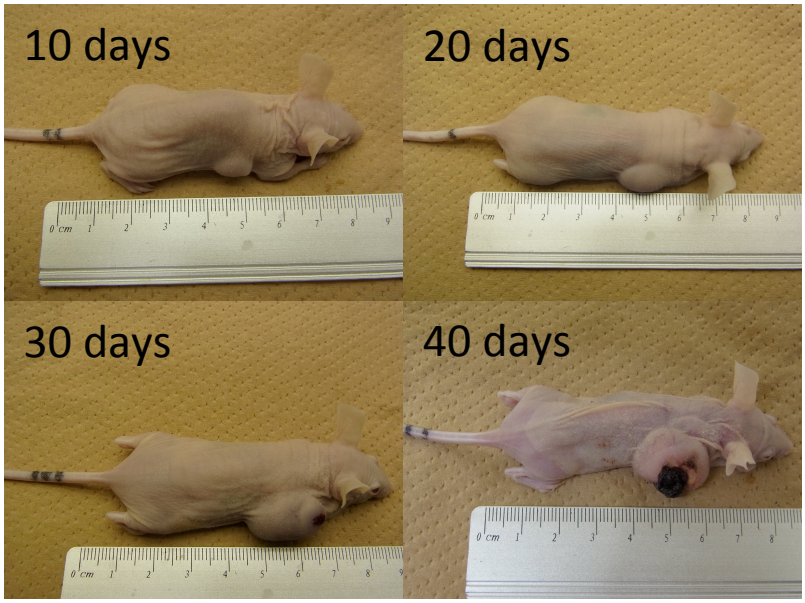
the expression of p-ERK that was highly expressed during the twenty to thirty days after transplantation, despite the PIs were similar. The high expression of p-ERK during that period might be caused by the cell cycle activity during the tumor progression.

In this section, tumorigenesis of CMM1 cells on xenografted nude mice with sufficient primary tumor growth and neovascularization was confirmed. The results obtained here indicate that it is appropriate to start the experimental treatment protocol from 10 days after transplantation till the end of the experimental period. The results here also indicate that the experimental period should be within 40 days after the xenograft because no severe tumor-related clinical sign and body weight loss were not observed in this period. In the next section, the effectiveness of single and combination use of low-dose cyclophosphamide and piroxicam was evaluated using this CMM1 cell xenografted mouse model.

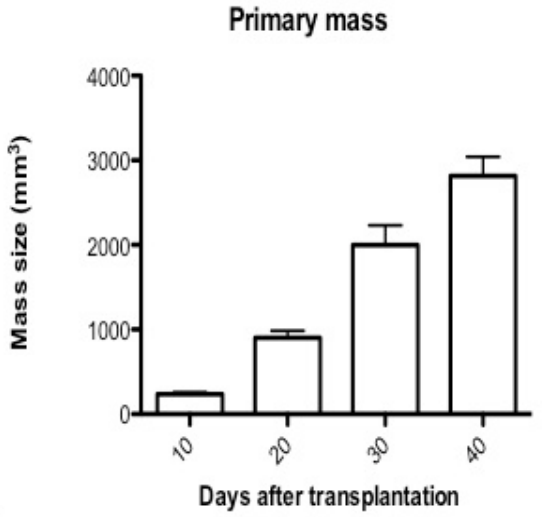
Table 2.1.1 Dilutions of each antibody for western blot analysis and immunohistochemistry.

Antibody	Dilution	
	WB	IHC
Actin	1: 10,000	-
COX-2	1: 1,000	-
ERK	1: 1,000	-
p-ERK	1: 2,000	-
VEGF	1: 300	1: 250
KI67	-	1: 100
CD31	-	1: 100

WB, western blot analysis; IHC, immunohistochemical staining



A.



B.

Fig. 2.1.1 Gross findings of primary tumor mass that subcutaneously developed in CMM1 xenografted nude mice at 10, 20, 30 and 40 days (A). Masses were rapidly developed in xenografted mice (B). Necrotic were found in the central area of masses after three and more weeks after transplantation.

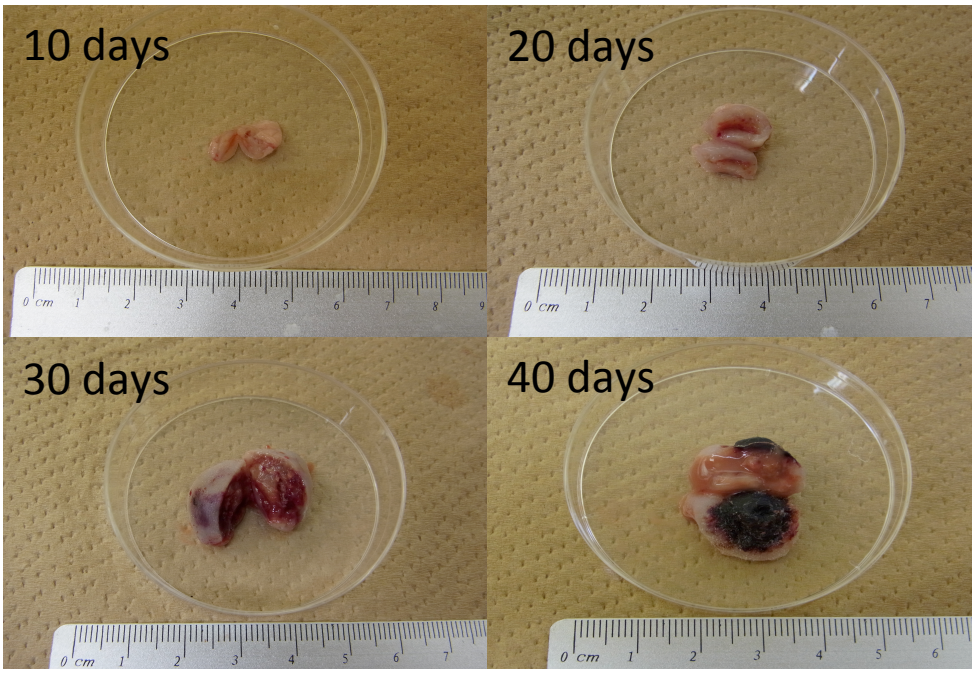
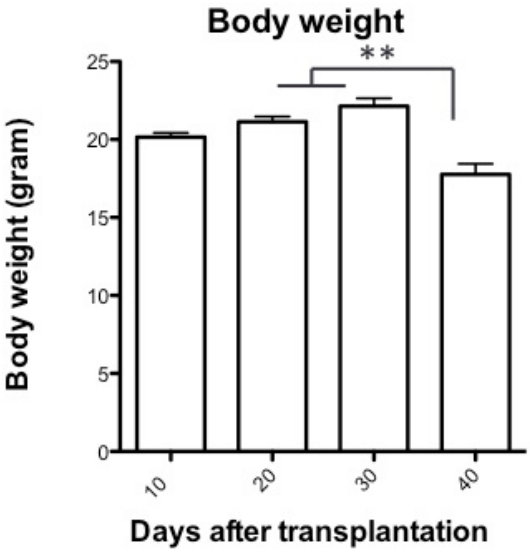


Fig. 2.1.2 Cut surface of primary tumor mass that subcutaneously developed in CMM1 cell-xenografted nude mice at 10, 20, 30 and 40 days.



$^{**}P < 0.01$

Fig. 2.1.3 Body weight of xenografted mice at each time point. At 40 days, body weights of xenografted mice were significantly lower than that of the 20 and 30 days ($P < 0.01$).

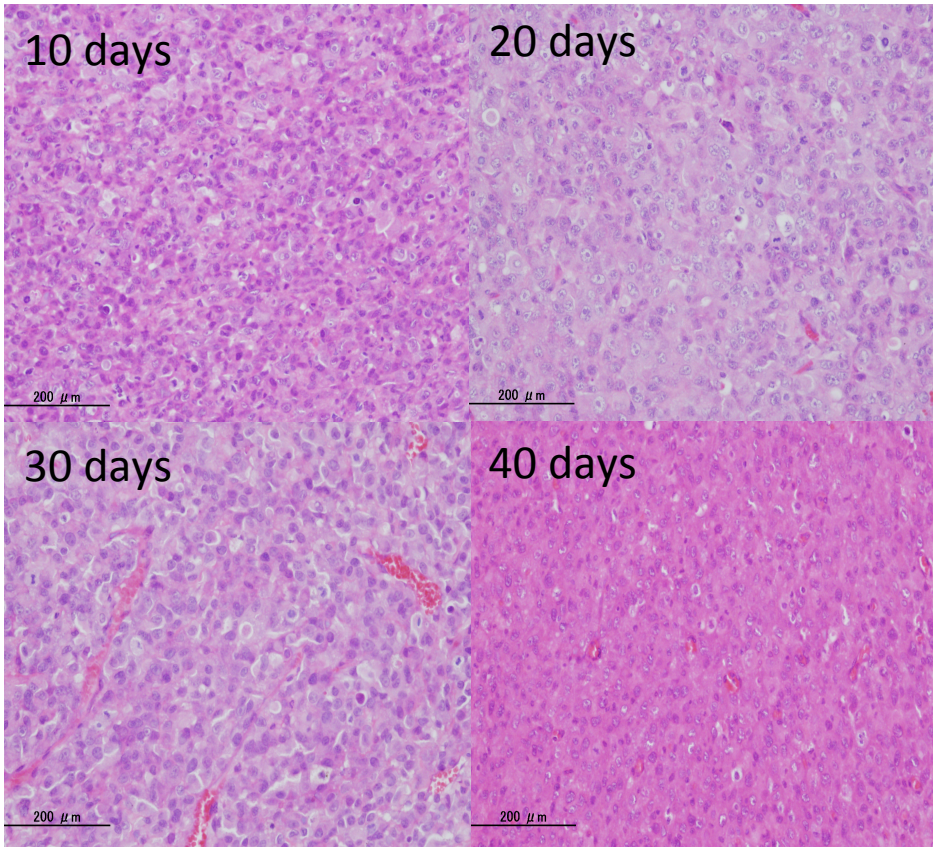
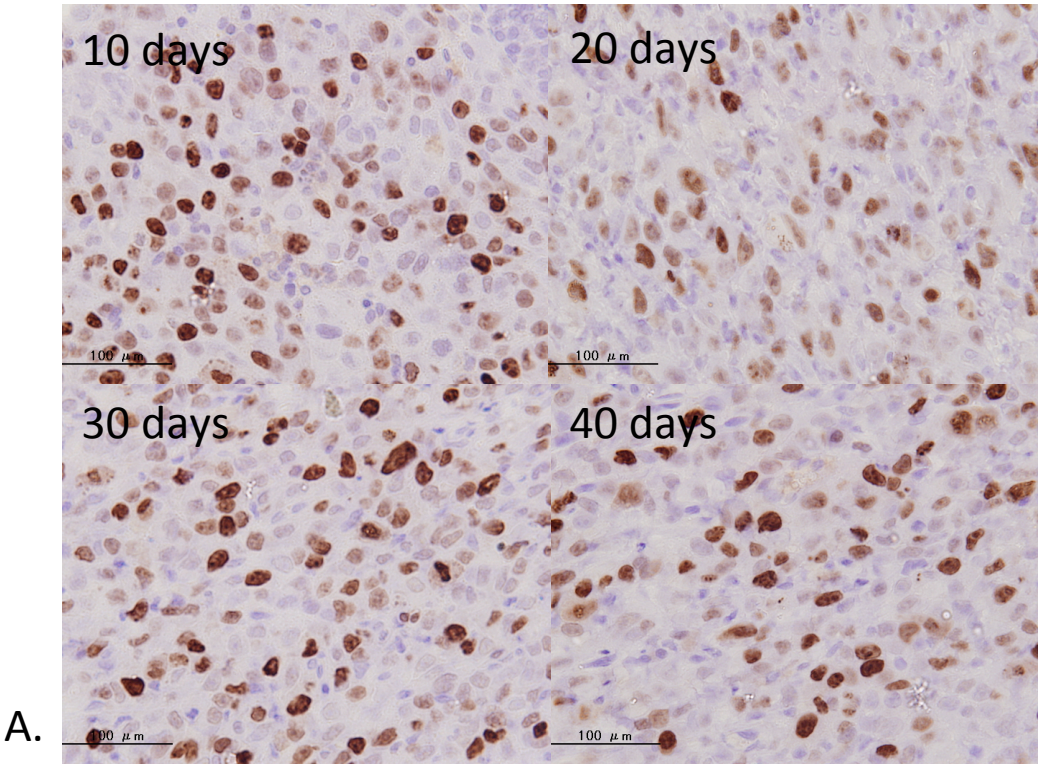
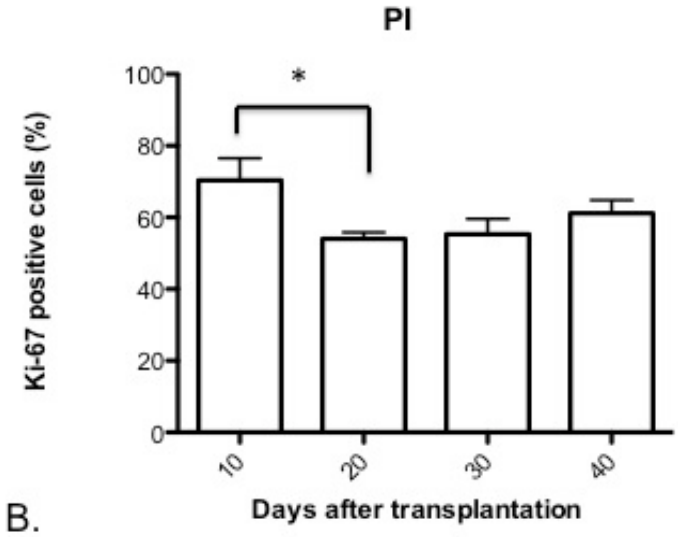


Fig. 2.1.4 Histopathological features of a primary tumor mass developing in the CMM1 cell-xenografted nude mice by hematoxylin-eosin staining. Histopathology of CMM1 tumor tissues showed sarcoma pattern. Tumor neovascularization was highly detected since 20 days.

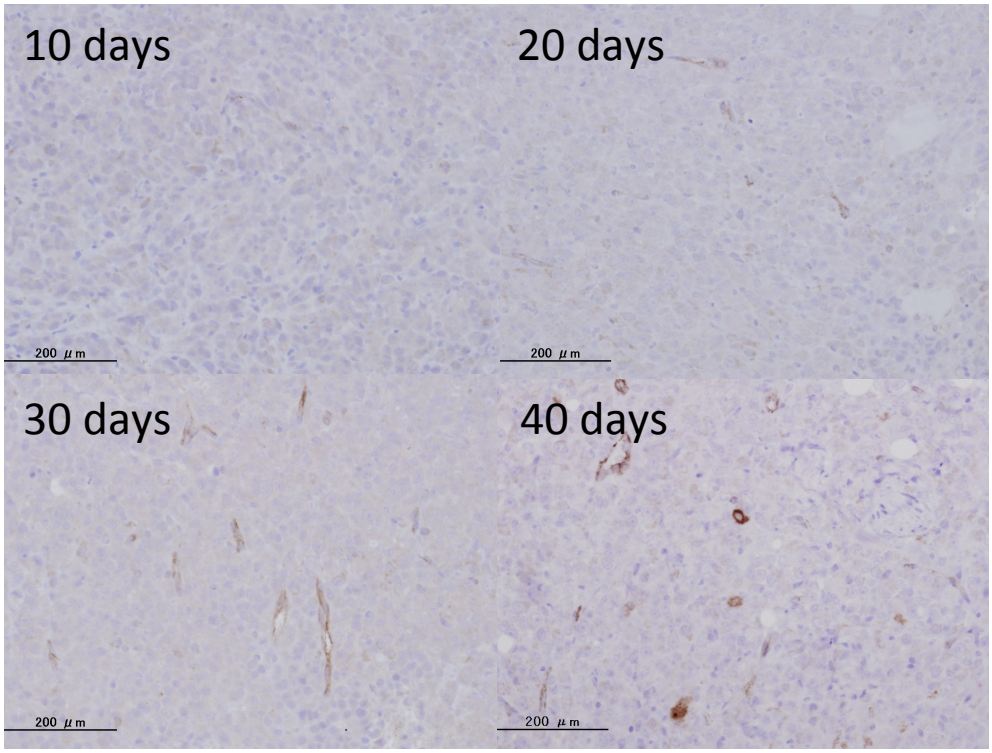




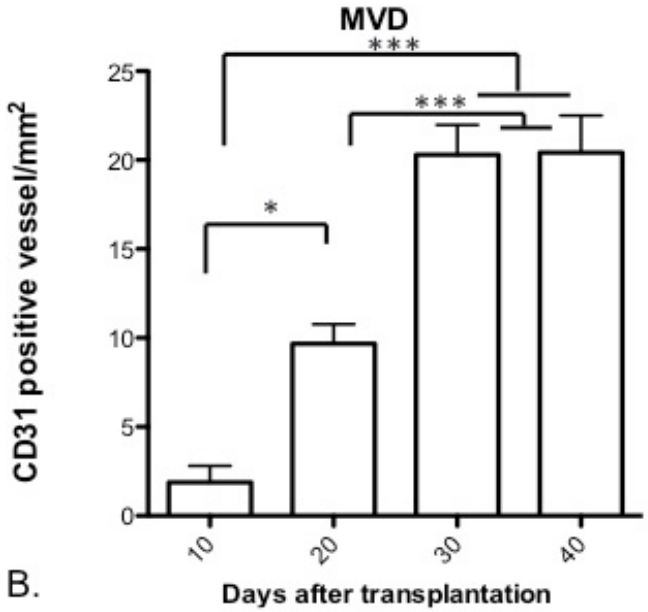
*$P < 0.05$

Fig. 2.1.5 Immunohistochemical staining for Ki-67 on CMM1 tumor mass (A). PI of tumor tissue at 10 days was significantly higher than that of 20 days. There were no significant differences among PI of 20 to 40 days group (B).

A.

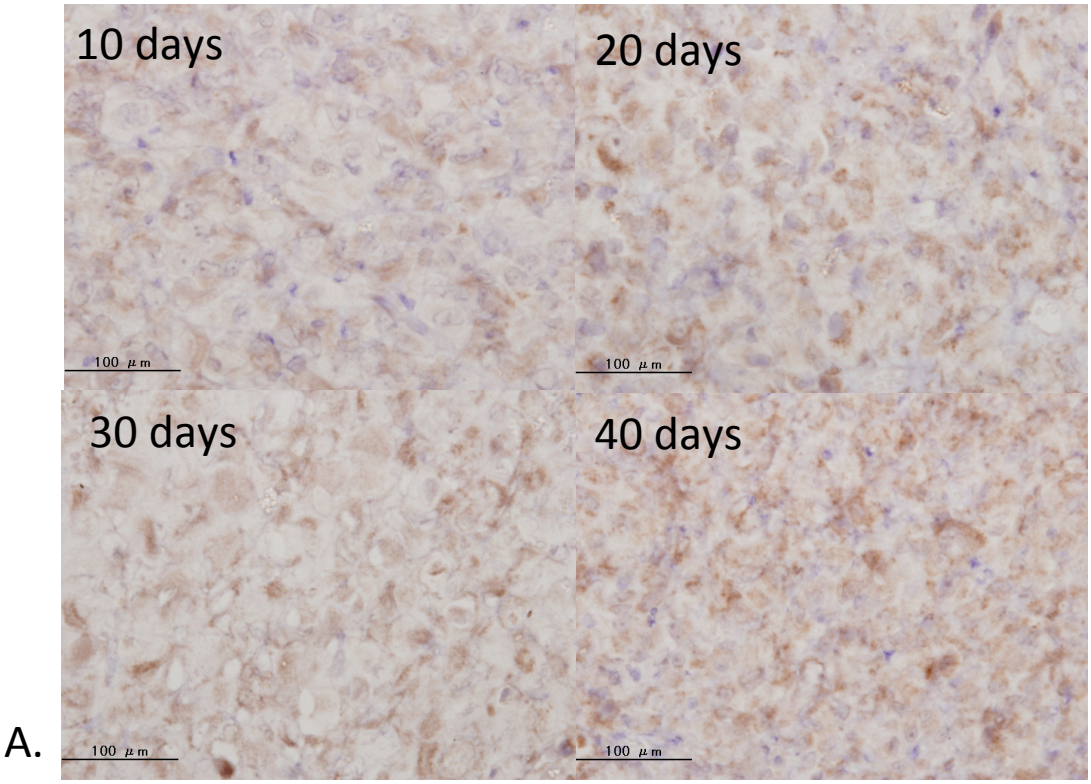


B.



*$P < 0.05$
***$P < 0.001$

Fig. 2.1.6 MVD expression of CMM1 tumor mass detected by immunohistochemical staining with CD31 (A). MVD gradually increased during 10 to 20 days and 20 to 30 days after transplantation ($P < 0.001$). Besides, MVD of primary tumor at 40 days was comparable to that of 30 days (B).



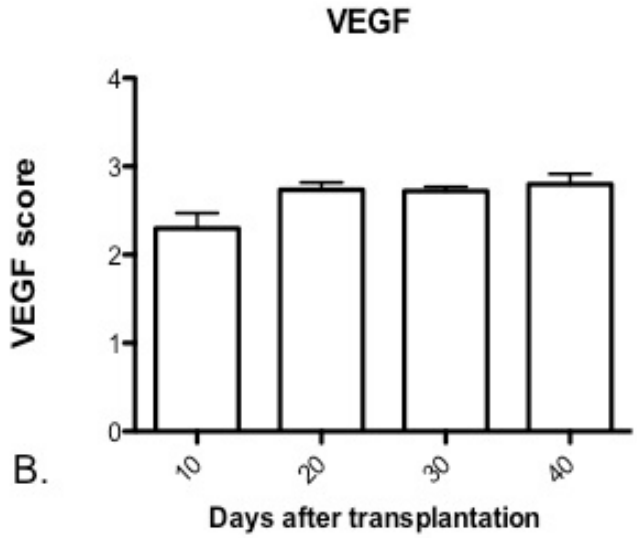


Fig. 2.1.7 Immunohistochemical staining for VEGF on CMM1 tumor mass. VEGF staining revealed two patterns, which were diffuse and granular patterns (A). VEGF score was medium to high level in all time points ($P < 0.05$).

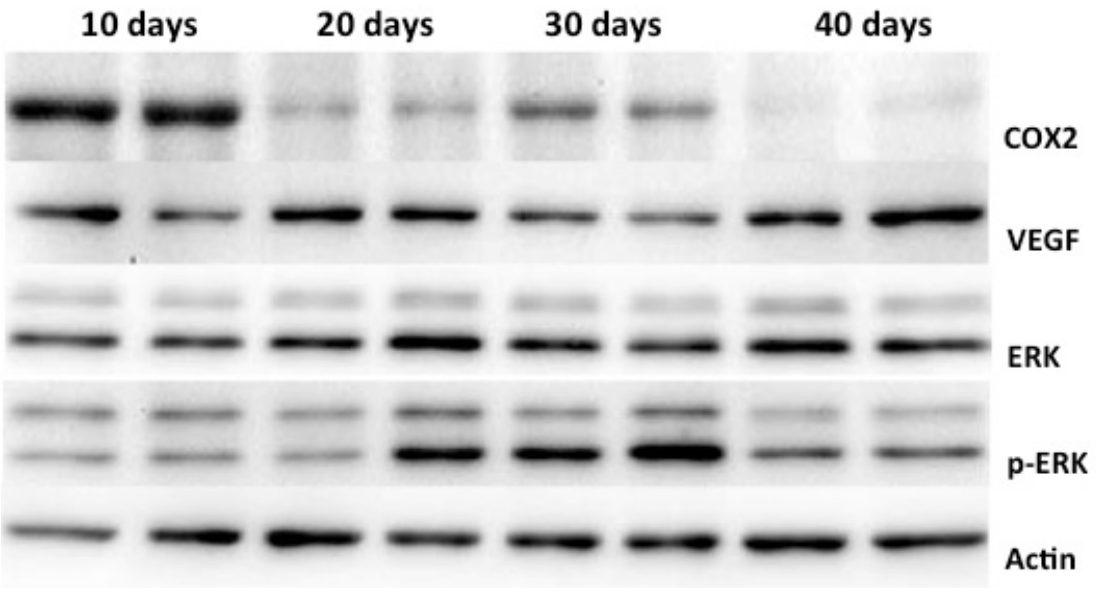


Fig. 2.1.8 Protein expressions in CMM1 tumor masses investigated by western blot analysis. COX-2 revealed high expression at 10 days after transplantation, while pro-angiogenic VEGF revealed medium expression during 10 to 30 days, and high expression at 40 days. The expression of ERK was medium to high in all time points, however, P-ERK expression revealed high expression during 20 to 30 days.