論文の内容の要旨

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**論文題目 The anti-tumour effects of etoposide on canine osteosarcoma.**
**(犬骨肉腫に対するエトポシドの抗腫瘍効果)**

Canine osteosarcoma (OSA) is the most common primary bone neoplasm affecting mainly large- and giant-breed dogs with an average age of 8 – 10 years and a subset of dogs less than 2 years of age. Wide marginal excision of the primary tumour via amputation or limb salvage procedures is the standard of care for appendicular OSA, but as most dogs with OSA have micrometastases at the time of presentation, the overall survival of dogs treated with surgery alone is only 11 – 21% at 1 year. Although combination treatment with surgery and adjuvant chemotherapy using doxorubicin, cisplatin or carboplatin improve the treatment outcome, most dogs with OSA ultimately die due to metastasis.

In addition, doxorubicin and cisplatin are contraindicated in patients with cardiovascular disease and renal insufficiency, respectively, which are conditions frequently diagnosed in older dogs. Carboplatin has a superior safety profile and may be a better candidate drug for canine OSA treatment; however, the high cost of this drug renders treatment to be expensive. For the past decades, advances in oncology research allowed better understanding of tumour biology and development of various novel cancer therapies. One of the major breakthroughs in cancer therapeutic is the discovery of molecular targeted drugs, which have demonstrated good anti-tumour effect against various malignancies. However, besides the high cost of these novel drugs, clinical efficacy against canine OSA has yet to be established and the prognosis of canine OSA remains universally poor. Since exploration and development of new drugs are time consuming and costly, there is an urgent need for effective alternative canine OSA therapeutic options using drugs that are readily available and of low cost.

Etoposide is a topoisomerase II inhibitor that is used extensively in human chemotherapeutic protocol against a wide range of neoplasms, including OSA. Investigation on its use in veterinary oncology is scarce, with some clinical studies suggesting its potential for clinical application in canine lymphoma and hemangiosarcoma patients. In a preliminary study conducted previously, canine OSA cell lines exhibited better sensitivity to topoisomerase inhibitors-induced anti-proliferative activity than canine mammary gland carcinoma and melanoma cell lines. Besides, canine OSA xenograft mouse models treated with etoposide had slower growing tumours than canine mammary gland carcinoma xenografts when exposed to the similar treatment regimen; however, the regimen employed in this study caused systemic toxicity.

Development of most tumours involves multiple pathways and genetic mutations. Consequently, multi-targeted anti-cancer therapies or combinational therapies may induce better response rate than

single-targeted therapies. Taking all these factors into account, the objectives of this study were to investigate the anti-tumour efficacy of etoposide on canine OSA, to identify novel combination therapeutic regimen for the treatment of canine OSA and the underlying molecular mechanism involved, and to establish a treatment protocol that can be translated into clinical application.

It has been reported that non-steroidal anti-inflammatory drugs (NSAIDs), such as piroxicam, exert anti-tumour properties. It has been reported to exert anti-tumourigenic activity against a range of canine malignancies and chemosensitise tumour cells to chemotherapy-induced cytotoxicity. Therefore, the effect of etoposide alone and in combination with piroxicam on canine OSA cells was investigated in chapter 1. Three canine OSA cell lines, namely HMPOS, POS and HOS, were exposed to etoposide alone or in combination with piroxicam at increasing concentrations. The effect of treatments on cell viability, survival ability, cell cycle distribution and apoptotic activity were evaluated. All canine OSA cell lines were sensitive to etoposide treatment at 0.2 μM, which is a concentration attainable *in vivo*. Etoposide alone significantly reduced viability and surviving fraction of all OSA cell lines. Exploration of the underlying mechanisms revealed that etoposide arrested cells at $G_2$/M phase by inhibiting tyrosine-dephosphorylation at Cdc2 (pCdc2-Y15), thus, inactivating the Cdc2-cyclin B1 complex, which is essential for transition from $G_2$ to mitotic phase. This subsequently triggered the apoptosis cascade, which was indicated by up-regulation of cleaved poly (ADP-ribose) polymerase (PARP), the hallmark of chemotherapy-induced apoptosis.

Whilst piroxicam alone did not show anti-proliferative effect, combination treatment with etoposide and piroxicam demonstrated better cytotoxic effect than either drug alone in a dose dependent manner. It was thought that piroxicam chemosensitised all OSA cell lines to the inhibitory effect of etoposide, and augmented the proportion of $G_2$/M and apoptotic cells. A significant discovery is that combination treatment down-regulated of survivin expression, an inhibitor of apoptosis protein (IAP), which was not observed in etoposide treatment alone. This was postulated to be one of the underlying mechanisms that contributed to the enhanced apoptotic activity and further up-regulation of cleaved PARP. Of note, these enhanced effects were evident only when piroxicam was supplemented at concentrations higher than 10 μM, which is the average serum concentration in dogs. Thus, it was hypothesised that the synergistic anti-proliferative effect might be absent *in vivo*. Considering cell culture experiments are unable to fully replicate the condition *in vivo*, further investigation using animal models is inevitable to validate the anti-tumour efficacy of etoposide alone and in combination with piroxicam against canine OSA. In addition, since suppression of survivin was associated with enhanced cytotoxicity in combination treatment, adjunct treatment with a survivin inhibitor may exhibit comparable inhibitory effects on canine OSA.

Survivin is abundantly expressed in a wide range of human and canine tumours and its expression is associated with unfavourable prognosis. Considering survivin plays a critical role in tumour progression, determining chemosensitivity and is preferentially expressed in cancer cells, it has been proposed as a cancer therapeutic target. Therefore, in chapter 2, the study on the effects of etoposide in combination with a survivin inhibitor molecule, YM155, on canine OSA cell lines was performed. Canine OSA cells that were exposed to either etoposide or YM155 alone or in combination were subjected to similar analytical tests as the previous chapter. YM155 suppressed survivin

expression and demonstrated potent anti-proliferative effect in all the cell lines at nanomolar concentration, which is within both canine and murine clinically attainable concentration. YM155 chemosensitised canine OSA cells to etoposide-induced cytotoxicity; however, the effect of treatment on cell cycle regulatory machineries was heterogenous.

Etoposide together with YM155 did not induce $G_2$/M arrest nor inactivation of the Cdc2-cyclin B1 complex, but altered cell cycle distribution primarily via enhancement of cell accumulation in S phase and reduction of $G_1$ cells in both POS and HOS cell lines, and had little effect on cell cycle distribution of HMPOS cell line. Survivin knock down by YM155 is known to sensitise cancer cells to chemotherapy-induced apoptosis. Surprisingly, combination treatment with etoposide and YM155 enhanced the apoptotic activity of HOS cell line only. The mechanisms of the synergistic effect on HMPOS and POS cell lines remain to be elucidated, but it was speculated that YM155 might target other signalling molecules besides survivin. The distinct genetic characteristics between different cell lines may have contributed to the heterogenous outcome observed in this study. Given the apparent synergistic effects demonstrated by this therapeutic combination, further investigation was conducted using xenograft mouse models to evaluate the anti-tumour efficacy and safety profile of this drug combination.

Then, the anti-neoplastic efficacy of etoposide alone and in combination with piroxicam was evaluated using xenograft mouse models in the first section of chapter 3. Five-week-old female BALB/c nu/nu mice was inoculated with HMPOS cells subcutaneously and were randomly assigned to control, piroxicam (0.3 mg/kg/day, p.o.), etoposide (20 mg/kg, i.p., q5d) and combination treatment groups (n = 6/group) 3 days later. Xenograft tumour samples that were collected at the experiment endpoint were analysed immunohistochemically for Ki-67 and survivin expression as well as for apoptotic cells using terminal deoxyribonucleotide transferase-mediated dUTP nick-end labeling (TUNEL) assay.

Both etoposide treatment alone and combination treatment with piroxicam delayed xenograft tumour progression predominantly through suppression of tumour cell proliferation. The average tumour volume of combination treatment group was the smallest but not markedly different from etoposide treatment group. Whilst the protein expression of survivin was reduced in these 2 groups, it was not accompanied with an increase in apoptotic activity, which is contrary to the findings in cell culture study. This could be due to the presence of anti-apoptotic machineries *in vivo*, abrogation of the apoptotic pathway by drugs or the time between tumour samples collection and etoposide administration.

These findings are in agreement with the hypothesis postulated earlier where addition of piroxicam into etoposide treatment regime did not further suppress tumour growth when compared with etoposide single agent treatment. Although piroxicam alone did not suppress tumour progression, prescription of piroxicam to canine OSA patients undergoing etoposide treatment protocol is tolerable since canine OSA is commonly associated with pain and this combination therapy did not cause adverse reaction. Thus, this therapeutic combination is a promising alternative therapy for canine OSA.

In the second section of chapter 3, the anti-tumour efficacy of etoposide in combination with YM155 was evaluated *in vivo*. Three days after inoculation of HMPOS cells subcutaneously, five-week

old BALB/c nu/nu mice were randomly assigned to control, YM155 (5 mg/kg/day, i.p., 5d/week), etoposide (20 mg/kg, i.p., q5d) and combination treatment groups (n = 6/group). Xenograft tumour samples were collected at the end of the experiment and subjected to immunohistochemical analyses as mentioned above. Combination treatment reduced tumour growth by 70%, primarily through alteration of the cell proliferation machineries, as evident in the decrease in Ki-67 protein expression. Surprisingly, the anti-neoplastic effect exhibited by etoposide and YM155 single agent treatments was negligible.

Besides, survivin expression was not suppressed following administration of YM155 alone, indicating further study is needed to establish a satisfactory treatment regimen that could suppress intra-tumoral survivin expression and aggravate the anti-tumour effect. The expression of survivin was the lowest in the combination treatment group; however, comparable with the findings in the previous section, survivin down-regulation did not consequent in elevated apoptotic activity. Taken together, these findings suggest that concomitant treatment with etoposide and YM155 may produce better response rate and is a potential novel therapeutic approach for canine OSA, but further optimisation of the treatment regimen is necessary before translation into clinical application.

In conclusion, this study revealed that etoposide exhibits effective anti-neoplastic activity against canine OSA both *in vitro* and *in vivo*. Adjunct treatment with piroxicam does not improve the anti-tumour effect of etoposide, but this combination therapeutic regimen is feasible as its analgesic property could improve the life quality of canine OSA patients without causing severe adverse reaction. Combination treatment with etoposide and a survivin inhibitor is a promising novel combination therapy for canine OSA as it exhibits superior anti-neoplastic effect than etoposide single agent treatment; however, more optimization work on the treatment regimen is required. Although the anti-neoplastic mechanisms of etoposide treatment either alone or in combination with piroxicam or survivin inhibitor have not been completely elucidated, modification in the cell cycle progression machineries is one of the mechanisms involved.