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Title	Analysis of tumor evasion mechanisms contributing to tumor radio-resistance and the radio-sensitizing effects of Janus kinase inhibitor oclacitinib in canine tumor cell lines [an abstract of entire text]
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Summary

Radiation therapy is a valuable treatment modality for cancer patients in both human beings and animals. In many patients, an initial course of radiation would exhibit a favorable response, whereas in most cases, the regrowth of tumor mass becomes a concern after radiation therapy. The regrowth of tumors after radiation therapy is thought to be due to complex mechanisms of it. Elucidating the mechanisms of tumor radio-resistance is required to establish a novel therapeutic strategy targeting the mechanisms of tumor radio-resistance.

The cytotoxic stress induced by radiation therapy activates T cell-mediating immune response in the tumor microenvironment (TME), leading to an effective antitumor response, but the expression of programmed death ligand 1 (PD-L1) on tumor cells, which is the ligand of programmed death 1 (PD-1) expressed on T cells, provides an immune evasion by suppressing cytotoxic T cells. Blockade of PD-1/PD-L1 axis in combination with radiation therapy enhanced antitumor effects of radiotherapy and improved treatment outcomes, indicating that the regulation of PD-L1 on tumor cells plays a pivotal role in overcoming tumor radio-resistance. Several regulatory mechanisms of PD-L1 expression have been shown in human tumors, however, little is known in canine tumors. In TME of human tumors, The Janus kinase (JAK)-signal transducer and activator of transcription (STAT) signaling pathway plays a key role in PD-L1 regulatory signaling.

Among JAK-STAT signaling pathways, STAT3 activation is well-known for its role in facilitating tumor growth. Irradiation in combination with JAK or STAT3 inhibitor enhanced radio-sensitivity and induced tumor growth delay in mice xenograft model of human cancer cells. Hence, Modulating the JAK-STAT signaling pathway may serve as an effective treatment strategy for overcoming tumor radio-resistance. The objectives of the present study were to elucidate the mechanisms of tumor radio-resistance focusing on the PD-L1 expression and the JAK-STAT signaling pathway in canine tumors, and to investigate the radio-sensitizing effects of oclacitinib for the establishment of novel therapeutic strategies to overcome tumor radio-resistance. In Chapter I, the effects of proinflammatory cytokines, IFN- γ and TNF- α were examined to demonstrate the upregulation mechanism of PD-L1 expression by these cytokines in canine malignant

melanoma cell lines and an osteosarcoma cell line. In Chapter II, radio-sensitizing effects of oclacitinib were evaluated both *in vitro* and *in vivo* using a canine osteosarcoma cell line and a canine malignant melanoma cell line. Furthermore, the mechanisms underlying the radio-sensitizing effects of oclacitinib were investigated by focusing on the STAT3 signaling pathway. Here this study demonstrates the mechanisms behind tumor radio-resistance with a particular emphasis on the PD-L1 expression and JAK-STAT signaling pathway, providing an opportunity to develop therapeutic agents targeting the JAK-STAT signaling pathway, including oclacitinib.

In Chapter I, to assess whether inflammatory signaling and DNA damage signaling are involved in PD-L1 regulation in canine tumors and elucidate the underlying mechanisms, the effects of interferon (IFN)- γ and tumor necrosis factor (TNF)- α treatment and X-irradiation exposure were evaluated in two canine malignant melanoma cell lines (CMeC and LMeC) and an osteosarcoma cell line (HMPOS). The protein level of PD-L1 expression was increased on all tumor cells treated with IFN- γ and TNF- α , but not with X-irradiation. Upon IFN- γ stimulation, higher gene expression of PD-L1, STAT1, STAT3, and genes regulated by STAT activation was demonstrated in all cell lines. Upregulation of these genes was inhibited by the addition of a JAK inhibitor, oclacitinib. In contrast, upon TNF- α stimulation, all cell lines indicated an increase in expression of the nuclear factor kappa B (NF- κ B) gene (RELA) and genes regulated by NF- κ B activation, whereas PD-L1 expression was upregulated in LMeC only. The addition of an NF- κ B inhibitor, BAY 11-7082 suppressed the upregulated expression of these genes. The upregulation of PD-L1 expression induced by IFN- γ and TNF- α treatment was reduced by oclacitinib and BAY 11-7082, respectively, indicating that PD-L1 upregulation by IFN- γ and TNF- α stimulation is regulated via the JAK-STAT and NF- κ B signaling pathways, respectively.

In Chapter II, to establish a therapeutic approach targeting the JAK-STAT signaling pathway, the radio-sensitizing effect of oclacitinib in canine tumors and the underlying mechanisms were assessed in HMPOS and CMeC cell lines. Oclacitinib significantly enhanced the radio-sensitivity of tumor cells both *in vitro* and *in vivo*. In cells treated with X-irradiation exposure, STAT3 expression was significantly activated in a dose- and time-dependent manner, which was effectively suppressed by oclacitinib. Oclacitinib significantly enhanced radiation-induced apoptosis by inhibiting the

expression of anti-apoptosis genes (*survivin*, *Bcl-x_L*, and *MCL1*) and Poly (ADP-ribose) polymerase (PARP) protein in HMPOS. Additionally, oclacitinib significantly inhibited the transcription of cell-cycle regulating genes (*CCND1*, *Cyclin-dependent kinase 4* and *6*) and arrested cell cycle progression from the G1 phase to subsequent phases. These results indicated that oclacitinib facilitated radio-sensitivity by triggering apoptosis and impeding cell cycle progression via STAT3 inhibition in canine tumor cell lines. This study suggested the clinical therapeutic potential of oclacitinib in combination with radiation therapy, aiming to enhance treatment efficacy and outcomes in dogs with tumors.

In conclusion, the regulatory mechanisms of PD-L1 expression on canine tumors were elucidated. The JAK-STAT signaling pathway could play a pivotal role in the regulation of not only PD-L1 upregulation induced by IFN- γ but also the radio-sensitivity of canine tumors. In addition, oclacitinib targeting STAT3 was shown to be effective as a radio-sensitizer in canine tumors.