Expression of vascular endothelial growth factor and its receptors in canine mast cell tumors

（犬の皮膚肥満細胞腫における血管内皮増殖因子およびその受容体の発現）

Summary of Doctoral Thesis

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Chapter 1. Introduction

Mast cell tumors (MCTs) are the most common cutaneous tumor in dogs. High malignant MCTs show the characteristic features of the immature mast cells (MCs), such as the decrease of the cytoplasmic granules, the increase of mitotic figures, and the high nuclear / cytoplasmic ratio, which are thought to be associated with malignant behaviors of MCT cells. Differentiation and maturation of skin MCs are mainly regurated by stem cell factor (SCF) and its receptor, c-Kit. However, c-Kit expression show no correlation with differentiation status in canine MCTs, indicating that other regulatory mechanisms contribute to the differentiation of the MCT cells. Vascular endothelial growth factor (VEGF) -A and its receptors (VEGFRs), Flt1 and Flk1, are key angiogenic factors and also essential for differentiation of hematopoietic lineages. Thus, VEGF-A, Flt1, and Flk1 are assumed to regulate differentiation of skin MCs and canine cutaneous MCTs.

Therefore, I aimed to clarify the contribution of VEGF-A and VEGFRs to differentiation and histological malignancy in MCT cells.

Chapter 2. Expression of VEGF-A and VEGFRs in canine cutaneous MCTs and their association with histological malignancy

In this chapter, I examined the correlation of expression of VEGF-A, Flt-1 and Flk-1 in tumor cells with histological grades determined by Patnaik (Grade I to III) and Kiupel (low and high-grades) grading systems and c-Kit immunoreactive patterns (c-Kit pattern I to III) in 135 MCTs. Furthermore, proliferative activity of MCT cells was assessed by Ki67 and proliferating cellular nuclear antigen (PCNA) immunohistochemistries.

VEGF-A expression in MCT cells was detected more frequently in Patnaik grade III, Kiupel high-grade and c-Kit pattern III MCTs. Flk1 expression were significantly associated with c-Kit patterns. In addition, co-expression of VEGF-A and Flk-1 showed significant correlation with all grading systems. However, Flt1 expression showed no correlation with histological malignancy. In normal canine skin, MCs exhibited VEGF-A

immunoreactivity in the cytoplasm and Flk1 immunoreactivity in the nucleus. However, MCT cells showed both cytoplasmic and nuclear localization of VEGF-A and Flk1 immunoreactivity. The results of this study indicate that VEGF-A and Flk1 expressed in MCT cells contribute to malignant progression of canine MCTs, especially via VEGF-A/Flk1 autocrine signaling. VEGF-A serves as a growth regulator of various neoplastic cells via Flk-1 in paracrine and autocrine loops. In normal MCs, VEGF-A/Flk1 signaling promotes cell migration. These findings indicate that VEGF-A/Flk1 signaling induce malignant phenotypes by promoting cell migration and proliferation in canine MCTs. In addition, nuclear VEGF-A is involved in the malignant changes and increase of cytoplasmic Flk1 enhanced cell survival by VEGF-A/Flk1 intracrine signaling in several tumors. Therefore, nuclear VEGF-A and cytoplasmic Flk1 observed in the tumor cells may also contribute to the MCT progression.

Chapter 3. Expression of VEGF-A and VEGFRs in canine cutaneous MCTs and their association with the differentiation of MCT cells

The aim of this study was to clarify the association of VEGF-A and Flk1 expression with differentiation status of tumor cells in canine cutaneous MCTs. In normal skin, immature MCs predominantly contain alcian blue $(AB)^+$ granules rather than safranin O $(SO)^+$ granules (AB>SO) in the cytoplasm. The MCs increase SO^+ granules with maturation and consequently differentiate to mature MCs containing predominant SO^+ cytoplasmic granules (AB<SO). In addition, expression of Gαi1 increased in MCs during maturation. I analyzed the association between expression of VEGF-A and Flk1 with differentiation of MCT cells determined by AB-SO staining and Gαi1 immunohistochemistry.

VEGF-A and/or Flk1 expression in the MCT cells were more frequently detected in AB>SO, AB-SO negative and Gαi1 negative MCTs. These findings indicate that VEGF-A and/or Flk1 expressing MCT cells maintain immature phenotypes. Thus, it is possible that VEGF-A/Flk1 downregulate differentiation of the tumor cells in canine cutaneous MCTs.

Chapter 4. Expression of VEGF-A and VEFRs in skin development.

The results of chapter 3 indicated that VEGF-A and Flk1 were involved in the regulation of the MCT differentiation. The aim of this study was to clarify the contribution of VEGF-A and Flk1 to differentiation of normal MCs in the skin. Expression of VEGF-A and VEGFRs were investigated in skin MCs of rats from 15 days of embryonic development (E15) to 90 days after birth (D90). Differentiation of MCs were assessed by AB-SO staining and c-Kit, MC Tryptase and Ki67 immunohistochemistries.

From E16 to D1, most of skin MCs were immature AB>SO cells. AB>SO MCs gradually decreased, while mature AB<SO MCs increased from Day 7 to 28. At D60 and D90, most of MCs showed AB>SO Flk1 and Flt-1 were expressed in most of MCs from Day 1 to 28, thereafter decreased to about 10% at Day 60 and 90. $Flk1^+$ MC proportions changed almost in parallel with the numbers of MCs and $Ki67^+$ MCs from E17 to Day 90. VEGF-A immunoreactivity was detected mainly in skin fibroblasts and keratinocytes, but in a few MCs from E17 to D90.

This study demonstrated the expression of Flk1 and Flt1 in differentiating MCs , but not mature MCs in the skin. Considering that the main function of Flt1 is suppression of Flk1 effects, VEGF-A/Flk1 and VEGF-A/Flt1 signaling may regulate the MC differentiation in a coordinated manner.

Chapter 5. Expression of VEGF-A and Flk1 in mouse bone marrow-derived MCs (mBMMCs) and their function on the differentiation of mBMMCs.

The results of chapter 2 to 4 lead to the possibility that VEGF-A and Flk1 regulate the differentiation of canine cutaneous MCTs and normal skin MCs. To clarify the effects of VEGF-A/Flk1 signaling on MC differentiation, I assessed the expression of VEGF-A and Flk1 during differentiation and maturation of MCs, and examine the changes of differentiation-related genes expression by VEGF-A and Flk-1 inhibitor using primary cultured mBMMCs.

Expression of *vegf-a* and *flk1* mRNA increased from 3 to 4 weeks, and thereafter decreased from 5 weeks of culture. In contrast, expression of *gata2* mRNA increased from 5 weeks of culture. Flk-1 inhibitor induced increase of *gata2* mRNA at 3.5 weeks of culture. GATA2 plays a crucial role for the MC fate decision and the maintenance of the cellular identity of the MCs. Therefore, results of this study indicate that VEGF-A/Flk1 signaling downregulate MC differentiation by inhibition of GATA2 expression. During differentiation of mBMMCs, *gata2* mRNA increased after decrease of *flk-1* and *vegf-a* mRNA expression, implying that VEGF-A/Flk-1 signaling downregulate GATA2 expression at early stage of MC differentiation, and thereafter GATA2 expression is increased by reduction of the VEGF-A/Flk-1 signaling.

Chapter 6. Conclusion

In this thesis, I aimed to clarify that the expression of VEGF-A and VEGFRs contribute to malignant progression and the differentiation of tumor cells in canine cutaneous MCTs. This study demonstrated that VEGF-A and Flk1 expression were more frequently detected in MCT cells showing histological malignancy and immature MC phenotypes such as AB-SO negative, AB>SO and Gαi1 negative. Flk1 was also expressed in immature MCs, but not in mature MCs in normal rat skin during development and growing periods. Moreover, the *in vitro* study using primary cultured MCs demonstrated upregulation of GATA2 expression by Flk-1 inhibitor, indicating that VEGF-A/Flk-1 signaling inhibited GATA2 expression during MC differentiation. Taken together, my results indicate that VEGF-A/Flk1 signaling suppresses the differentiation of tumor cells by inhibition of GATA2 expression and maintains the phenotypes of immature MCs, which lead to the malignant progression in canine cutaneous MCTs. Treatment targeted at VEGF-A/Flk-1 signaling would provide a new therapeutic strategy of the highly malignant MCTs.