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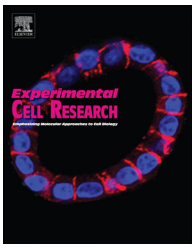

Research Article

Interleukin-8 promotes canine hemangiosarcoma growth by regulating the tumor microenvironment

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ABSTRACT

Interleukin-8 (IL-8) gene expression is highly up-regulated in canine hemangiosarcoma (HSA); however, its role in the pathogenesis of this disease is unknown. We investigated the expression of IL-8 in canine HSA tissues and cell lines, as well and the effects of IL-8 on canine HSA *in vitro*, and *in vivo* using a mouse xenograft model for the latter. Constitutive expression of IL-8 mRNA, IL-8 protein, and IL-8 receptor were variable among different tumor samples and cell lines, but they showed stable steady states in each cell line. Upon the addition of IL-8, HSA cells showed transient intracellular calcium fluxes, suggesting that their IL-8 receptors are functional and that IL-8 binding activates relevant signaling pathways. Yet, neither addition of exogenous IL-8 nor blockade of endogenous IL-8 by neutralizing anti-IL-8 antibody (α-IL-8 Ab) affected HSA cell proliferation or survival *in vitro*. To assess potential effects of IL-8 in other tumor constituents, we stratified HSA cell lines and whole tumor samples into "IL-8 high" and "IL-8 low" groups. Genome-wide gene expression profiling showed that samples in the "IL-8 high" tumor group were enriched for genes associated with a "reactive microenvironment," including activation of coagulation, inflammation, and fibrosis networks. Based on these findings, we hypothesized that the effects of IL-8 on these tumors were mostly indirect, regulating interactions with the microenvironment. This hypothesis was supported by *in vivo* xenograft experiments where survival and engraftment of tumor cells was inhibited by administration of neutralizing α-IL-8 Ab. Together, our results suggest that IL-8 contributes to establishing a permissive microenvironment during the early stages of tumorigenesis in HSA.

Abbreviations: HSA, hemangiosarcoma; IL-8, interleukin-8; IL-8R, interleukin-8 receptor; α-IL-8 Ab, anti-IL-8 antibody; IPA, ingenuity pathway analysis; GFP, green fluorescent protein

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Introduction

Hemangiosarcoma (HSA), which occurs commonly in dogs, is a highly metastatic and almost always incurable cancer with extensive vascular networks where cells form abnormal, distorted blood vessels, capillaries, and sinusoids [1,2]. This cancer is a heterogeneous disease, and its precise origins still remain to be elucidated [3]. Treatment options for this disease are limited and outcomes are generally unrewarding [4], as over 50% of dogs affected with this disease still die within 6 months of diagnosis, and clinical trials using new combinations of old drugs [5] and different treatment modalities [6] have shown no benefit over the standard of care. The lack of effective treatments is at least partly due to the fact that the pathogenesis and biological features of canine HSA are incompletely understood.

IL-8, also known as CXCL8, is a pro-inflammatory and pro-angiogenic CXC chemokine, which is mainly known to promote neutrophil chemotaxis and degranulation [7]. IL-8 is produced by fibroblasts, endothelial cells, and monocytes during stimulation, but constitutive expression in these normal cells is negligible [8]. In cancer, high expression of IL-8 is frequently observed in a range of tumor types, and its effects on cell proliferation and survival have been demonstrated in human cancers such as breast, gastric, colon, pancreatic, ovarian, bladder, prostate, and melanoma [7]. The cancer cells secreting IL-8 proliferate and survive through autocrine IL-8 signaling pathways, including activation of Akt [9] and through regulation of the activity of the mitogen-activated protein kinase signaling cascade [10,11]. In addition, cancer-derived IL-8 promotes not only cell invasion and migration but also metastasis by inducing neutrophil infiltration and tumor-associated macrophage production of growth factors at the tumor site [12–14]. Furthermore, IL-8 is associated with angiogenesis in various human tumors, suggesting this cytokine might be an important regulatory factor in the tumor microenvironment [7,15,16].

Previously, we used genome-wide profiling to discover that inflammation and angiogenesis are critical features in the pathogenesis of canine HSA [17]. More specifically, we showed that IL-8 was highly up-regulated in canine HSA cells compared to non-malignant endothelial cells derived from splenic hematomas, suggesting that IL-8 has a crucial role in the malignancy of these tumors [17]. Recent studies also have shown that the levels of IL-8 were higher in blood obtained from dogs with mammary cancer than in blood from healthy dogs, suggesting that IL-8 may be an important prognostic serum marker [18,19]. However, a functional role of IL-8 in spontaneous canine cancers has not been reported.

The aims of this study were to examine the constitutive expression of IL-8 and to explore its functional roles in canine HSA in terms of direct effects on cells *in vitro* and/or modulation of the tumor microenvironment *in vivo*. We analyzed genome-wide gene expression profiling in canine HSA samples, and then showed that IL-8 promotes HSA growth by modulating the tumor microenvironment *in vivo*.

Materials and methods

Samples and histopathological examination

Samples were obtained from client-owned dogs diagnosed with spontaneous HSA as described [20]. Demographic and clinicopathologic data including age, breed, sex, and affected site for each dog are shown in Supplementary Table 1. The metastatic status for every dog in this group was either confirmed positive ($n=14$) or uncertain ($n=11$), where "uncertain" means the dog was euthanized at diagnosis, a necropsy was not done, or the case was lost to follow up. Samples from twenty-four primary or metastatic tumor tissues were formalin-fixed and paraffin embedded. Sections (4 μm) were stained with hematoxylin and eosin (H&E) for histopathological evaluation to confirm the diagnosis. Semi-quantitative inflammation scores were assigned to each sample based on cellular infiltrates (myeloid and mononuclear cells), necrosis, and fibrosis. Samples with no inflammation were scored as 0, and those with mild, moderate, and severe inflammation were scored respectively as 1, 2, and 3 as previously described [21]. Twelve cell lines were used for this study. The characteristics of the DD-1, Dal-4, SB, FH, GM, and EFS/EFB cell lines were described previously [3,20,22]. The JHE, JSP, JLI, and JLU cell lines were established from tissues of one dog with disseminated HSA (heart, spleen, liver, and lung), and the SH cell line was established from a splenic HSA using previously described methods [17,22]. Newly acquired samples were obtained with owner consent and with approval of the University of Minnesota Institutional Animal Care and Use Committees (protocols 0802A27363 and 1101A94713).

Cell culture

Seven of the HSA cell lines (JHE, JLI, JLU, JSP, COSB, Dal-4, and DD-1) were derived from the tumors of four dogs diagnosed with HSA. Four of these cell lines (JHE, JLI, JLU, and JSP) were obtained from tumor tissues in different distant organs from the same dog. These HSA cells were cultured as described previously [3,20,22] and used for the *in vitro* experiments in this study. COSB was a low passage derivative of the SB cell line isolated from a mouse xenograft.

Genome-wide gene expression profiling

Twenty-four tumor tissue samples ($n=24$) and twelve cell lines ($n=12$) were used for genome-wide gene expression profiling (Supplementary Table 1). RNA was isolated using the TriPure Isolation Reagent (Roche Applied Science, Indianapolis, IN, USA) and the RNeasy Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's instructions. RNA from the samples was quantified and assessed for quality. Briefly, samples determined to be suitable for analysis based on RNA quality (ratio of absorbance at 260 nm over 280 nm between 1.95 and 2.1 and Bioanalyzer RNA Integrity Number >6.1) were labeled using Agilent's Microarray One-Color Low-Input Quick Amp Labeling kit, hybridized to Agilent canine $4\times44{,}000$ feature gene chips according to Agilent's Protocol Version 6, and read using an Agilent array scanner (Agilent, Santa Clara, CA, USA). Bioanalyzer quality control, RNA labeling, and microarray hybridization were performed by the BioMedical Genomics Core of the University of Minnesota.

After microarray hybridization, Agilent quality control algorithms in Expressionist Refiner Module (v. 7.5; Genedata, Basel, Switzerland,) were used to confirm that data from each chip met the manufacturer's standards for quality control and quality assurance. Of 45,220 features on each array, 35,676 that had annotation to known genes were used for analysis. Annotated