Evaluation of dysregulation of the receptor tyrosine kinases Kit, Flt3, and Met in histiocytic sarcomas of dogs

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Objectives—To evaluate canine histiocytic sarcoma cell lines and tumor samples for dysregulation of the Kit/stem-cell factor (SCF), Flt3/Flt3 ligand (Flt3L), and Met/hepatocyte growth factor (HGF) receptor tyrosine kinase signaling pathways, as these are known to contribute to the differentiation and survival of normal dendritic cells as well as malignant transformation of dendritic cells in mouse models.

Sample Population—4 histiocytic sarcoma tumor cell lines and 35 formalin-fixed histiocytic sarcoma specimens obtained from dogs.

Procedure—Histiocytic sarcoma cell lines were evaluated for expression of Kit/SCF, Flt3/Flt3L, and Met/HGF by use of reverse transcriptase-PCR procedures. Histiocytic sarcoma cell lines and tumor samples were evaluated for mutations in Kit, Flt3, and Met by use of PCR analysis of genomic DNA, followed by both sequencing and fluorescent PAGE for deletions or internal tandem duplications. The ability of the multitargeted split-kinase inhibitor SU11654 to block proliferation and induce apoptosis of histiocytic sarcoma cell lines was also evaluated.

Results—No mutations in Kit, Flt3, and Met were identified in any of the cell lines or tumor samples evaluated. Furthermore, SU11654 did not induce cell-cycle arrest or apoptosis of histiocytic sarcoma cell lines, even at supratherapeutic doses.

Conclusions and Clinical Relevance—These data suggest that dysregulation of Kit/SCF, Flt3/Flt3L, and Met/HGF signaling pathways is unlikely to occur in histiocytic sarcomas of dogs and that inhibitors of the Kit, Flt3, and Met pathways are unlikely to provide clinical benefit to dogs with histiocytic sarcomas.

Histiocytic sarcoma (also known as malignant histiocytosis) is a neoplastic disorder arising from cells of the histiocytic lineage (macrophages and dendritic cells). The disease is characterized by infiltration of many organs including the spleen, liver, lungs, lymph nodes, and bone marrow. Breeds of dog known to be at increased risk for this disease include Rottweilers, Golden Retrievers, Flat-Coated Retrievers, and Bernese Mountain Dogs. Diagnosis of histiocytic sarcoma is usu-
domain point mutations have been identified in Flt3; these mutations are known to be carried in the Met is aberrantly expressed or mutated and some of kinase domains separated by a kinase insert. The ligands for Kit and Flt3 are SCF and Flt3L, respectively. Both Kit and Flt3 play important roles in the survival and self-renewal of early hematopoietic progenitors and monocytic precursors (particularly Flt3) and in early lymphoid development; Flt3 signaling is critical in the development of dendritic cells from hematopoietic cells.

Both Kit and Flt3 are proto-oncogenes, and when constitutively activated, they can cause malignant transformation. Indeed, expression of constitutively active Kit or Flt3 in murine hematopoietic progenitors results in the development of hematologic malignancies. In naturally occurring cancers, mutations in Kit and Flt3 are detected in a variety of different neoplastic disorders. Deletions in the juxtamembrane domain of Kit leading to receptor activation are detected in 30% to 60% of gastrointestinal stromal tumors in humans. Aggressive mastocytosis in humans is often associated with the presence of an activating point mutation in the kinase domain (catalytic domain) of Kit. In contrast, approximately 30% to 50% of high-grade canine mast cell tumors possess internal tandem duplications in the juxtamembrane domain, which also result in dysregulation of Kit function. Similar juxtamembrane domain duplications and catalytic domain point mutations have been identified in Flt3 from humans with acute myeloid leukemia. Lastly, both Kit and Flt3 are aberrantly expressed in several human neoplasms, including lung carcinoma, glioblastoma, ovarian tumors, leukemias, and lymphomas.

Another RTK intimately associated with neoplasia is Met, which is normally expressed on a wide variety of epithelial cells; its ligand, HGF, is primarily produced by stromal cells. Interactions of Met and HGF promote an array of cellular responses, such as proliferation, scattering (motility), and branching morphogenesis, which are all important in normal processes such as wound healing, angiogenesis, and cell invasion and differentiation. In several human carcinomas, Met is aberrantly expressed or mutated and some of these mutations are known to be carried in the germline. Results of recent studies have indicated that mice carrying mutations in the Met kinase domain develop histiocytic sarcoma as well as other forms of neoplasia, suggesting a relationship between Met dysfunction and malignant transformation of dendritic cells. Moreover, aberrant Met expression has been described in cancers in dogs, indicating that it is likely that dysfunction of this pathway also contributes to cancer development in this species.

Given the fact that Kit and Flt3 are important for normal histiocyte differentiation and that dysregulation of Kit, Flt3, or Met can lead to the development of neoplastic disease, it was logical to suspect that these genes may be mutated in canine histiocytic sarcoma. Therefore, the purpose of the study reported here was to evaluate canine histiocytic sarcoma cell lines and tumor samples for dysregulation in the Kit/SCF, Flt3/Flt3L, and Met/HGF signaling pathways in an effort to determine whether inhibitors of these RTKs would be useful for the treatment of this disease.

**Materials and Methods**

**Reagents and cell lines**—Cell lines (provided by one of the authors) used in this study included 030210, DH82, and Nike (all of which are canine histiocytic sarcoma cell lines) and ML3 (a myeloid leukemia cell line). These cell lines were established and characterized previously in the laboratory of one of the authors (PFM). A C57 mouse mast cell line and C2 canine mast cell line were also used. The Madin-Darby canine kidney cell line and the mouse P815 mastocytoma line were purchased from American Type Culture Collection. All cell lines were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum, nonessential amino acids, sodium pyruvate, HEPES, penicillin, streptomycin, and t-glutamine. A new histiocytic canine cell line (Taz) was established in our laboratory from a tumor histiocytic sarcoma resected from a dog. All of the histiocytic cell lines were confirmed to be of histiocytic origin by use of immunohistochemical staining (to identify CD1, CD11c, and ICAM-1). The split-kinase inhibitor SU11564 and Met inhibitor PHA665752 were also used. Fresh stock solutions of inhibitor (10mM) were prepared before each experiment by dissolving approximately 4 mg of inhibitor in 1 mL of dimethyl sulfoxide; this stock solution was then used for further dilution in dimethyl sulfoxide. For flow cytometry, the A435 anti-Kit monoclonal antibody conjugated to phycoerythrin was used at a dilution of 1:200.

**Histiocytic sarcoma tissue samples**—Histiocytic sarcoma tissue samples were obtained from a large bank of previously characterized tumors. Full records of each dog's history were reviewed for relevance to the screening. All of the tissues were formalin fixed and embedded in paraffin at the Veterinary Teaching Hospital of the University of California, Davis. Forty-one samples were available for use: 36 yielded suitable gDNA. Dog breeds represented included Bernese Mountain Dogs (n = 22), Rottweilers (4), Golden Retrievers (3), Flat-Coated Retrievers (3), Poodle (1), and Bassett Hound (1); breed was unknown for 2 dogs. Within this population, there were 7 sexually intact males, 7 sexually intact females, and 14 neutered females; sex was unknown for 1 dog. The median age was 7 years (age range, 3 to 12 years).

**Cloning of canine Flt3**—Canine Flt3 cDNA was cloned from the canine myeloid leukemia cell line ML3. Starting primers were placed in regions of high homology between human and mouse sequences. The resultant products were cloned into the pGEM-T vector and directly sequenced.

**RT-PCR assays for receptor tyrosine kinases and ligands**—Cell lines were evaluated for expression of the Kit/SCF, Flt3/Flt3L, and Met/HGF by use of the following primer pairs: SCF (sense 5'-GAGCATATGAAAGGGA TTCGCCGGA-3' and antisense 5'-CACCCCGGGTTAG-CGAAGGGGGTAAACC-3'); Kit (sense 5'-CCCATGCGGGGGGTATG-TATGAAGTACAGTGGAAG-3' and antisense 5'-CTGATTAAGTGGTATGCGCCCATG-3'); Flt3L (sense 5'-GGCAGGCGCAGGCTGGACCCAA-3' and antisense 5'-CCCTGACAGGGCGACAGTGAC-3'); Flt3 (sense 5'-
CCAGTCGGCGGTGGCGCGCCGGCCGGCCGGGTACG-3' and antisense 5'-TCAGGGACTGGTGGGATTTAAAGACCCA-
GAG-3'; HGF (sense 5'-GGTGCTATACTCTTGGACCCCTG-3' and antisense 5'-CCAGGGAGTTAGTGGGCTATCG-3'); Met (sense 5'-GGACTTTTCCTGTGGCTGAAGG-3' and antisense 5'-GGAGTCTGACGATCTTG-3'); and GAPDH (sense 5'-GCTGCTTTAATCTGGCAAGG-3' and antisense 5'-GAATACATGTCGAACATGTCG-3'). All primer pairs were designed to span at least 1 intron, thereby making identification of gDNA contamination in the reaction assay easy to identify. For RT-PCR assay of cell lines to detect mRNA for Met, HGF, and HGFA, total RNA was extracted from 20 x 10^6 cells by use of standard phenol and guanidine isothiocyanate purification and cDNA was synthesized according to the manufacturer's specifications. Polymerase chain reaction analysis was performed for 40 cycles at 94°C for 30 seconds, 60°C for 30 seconds, and 72°C for 90 seconds. For all PCR analyses, GAPDH was used as a positive control.

**Strand analysis of RTKs**—Isolation of the gDNA from the paraffin-embedded tissue blocks was performed following an established protocol. In brief, two 25-µm slices of the block were placed in DNase-free tubes. Two washes with xylene and 100% ethanol were completed, and the pellets were air-dried. Digestion was undertaken with proteinase K buffer for approximately 5 hours at 55°C. The gDNA was isolated by use of a standard isolation column according to the manufacturer's specifications with 150 µL of nuclelease-free water. The resultant DNA samples were quantitated via spectrophotometry. The primers used for PCR amplification of gDNA for fluorescent PAGE were as follows: Kit exon 11 (sense 5'-CCCCACTGTA-
GAATCAGTTAGGGAAG-3' and antisense 5'-GGAGTCTGACGATCTTG-3'); Kit exon 17 (sense 5'-TGATATCAGACAGCTTGGCTG-3' and antisense 5'-GGTTCTTTTGACCATGAT-3'); Flt3 exon 11 (sense 5'-GCAATTTAGGTAGTAAAGCCAG-3' and antisense 5'-GAAACA TTGGGACCTTTGACCACAT-3'); Flt3 exon 11 (sense 5'-GCCATCCACTTCATTCGAGTC-3'); and Flt3 exon 17 (sense 5'-GAGACCTGCTGCTTTTAACTCTGGCAAAG-3' and antisense 5'-GCTGCTTTTAACTCTGGCAAAG-3'). The PCR products were evaluated via agarose gel electrophoresis and then directly sequenced at the University of California-Davis Sequence Facility.

**Flow cytometry**—Cell lines were counted and then resuspended in 24-well plates at a concentration of 0.25 to 0.5 x 10^6 cells/well (depending on growth rates) in complete medium. Cells were left untreated, or SU11654 was added to the wells at a final concentration of 0.01 µM (sub-therapeutic dose) or 0.1 µM (therapeutic dose). After 72 hours, plates were collected and the WST-1 (tetrazolium salt that is cleaved to formazan dye by metabolically active cells) assay was performed (according to the manufacturer's specifications) to determine the relative cell number in each well. Absorbency readings were performed on an ELISA plate reader after 5 minutes of incubation. To evaluate the effect of Met inhibition, cells were plated in the aforementioned manner and then left untreated, cultured with rhHGF (50 ng/mL), or cultured with rhHGF and PHA665752 (0.1 µM) together. After 72 hours, plates were collected and the WST-1 assay was performed according to the manufacturer's specifications to determine the relative cell number in each well. Absorbency readings were performed on an ELISA plate reader after 5 minutes of incubation. These experiments were performed in triplicate. The P815 and MDCK cell lines were used as positive control samples for these experiments.

**Results**

Cloning of canine Flt3—To begin to assess the roles of Kit/SCF, Flt3/Flt3L, and Met/HGF signal transduction in histiocytic sarcomas in dogs, it was first necessary to determine whether these genes are expressed in the relevant cell lines. Although sequences for canine Kit, SCF, Flt3L, Met, and HGF were available in GenBank (AF090030, S533329, AF155148, AY543631, and AY543632, respectively), canine Flt3 had not yet been cloned and the canine genome was not yet available. Therefore, we cloned the gene from the canine acute myeloid leukemia cell line ML3, initially using primers derived from regions of homology between the mouse and human sequences to generate PCR products, and then using primers designed from these PCR products to complete the entire sequencing. Canine Flt3 is composed of 994 amino acids (GenBank No. DQ007998) and is located on canine chromosome 25 (CanFam 1.0). It has 90% amino acid homology with human Flt3 and 80% amino acid homology with mouse Flt3. As expected, critical domains of Flt3, such as the juxtamembrane and kinase domains, have 100% homology with both species.

**Expression of Kit/SCF, Flt3/Flt3L, and Met/HGF in canine histiocytic sarcoma cell lines**—Because of the possible role of Kit, Flt3, and Met dysregulation in the malignant transformation of dendritic cells, canine histiocytic sarcoma tumor cell lines were assessed for...
expression of these RTKs and their respective ligands. All cell lines expressed mRNA for Kit, Flt3, Met, SCF, and Flt3L (Figure 1). Additionally, all lines except the 0302 line also expressed mRNA for HGF. Incidentally, this was also the only cell line that expressed low levels of mRNA for Met. Antibodies were available for identification of Kit via flow cytometry, and protein could be readily detected on the cell surface of all histiocytic sarcoma lines (Figure 2). Interestingly, the cell lines had variable levels of Kit expression; the functional consequences of these differences are not currently known. Because of a lack of reagents with which to identify the other proteins via flow cytometry, protein expression of Flt or Met was not assessed. Nevertheless, data from the canine histiocytic tumor cell lines investigated indicate that Kit/SCF, Flt3/Flt3L, and Met/HGF pathways are present in histiocytic sarcomas in dogs.

Analysis of cell lines and tumor samples for Kit, Flt3, and Met mutation—To begin to assess the potential role of Kit, Flt3, or Met dysregulation in canine histiocytic sarcoma, we evaluated these genes for evidence of mutation in regions known to frequently undergo mutation in human and canine neoplasms. For Kit and Flt3, a previously described technique termed strand analysis was used to evaluate these genes for evidence of duplications or deletions in exons 11 and 17. Briefly, this analysis involves PAGE of fluorescently labeled PCR products that can detect insertions or deletions of 1 to 2 or more base pairs and provides rapid screening of several samples simultaneously. We have recently used this technique to screen canine mast cell tumors for Kit internal tandem duplications in exon 11 and have found it to be reliable, accurate, and efficient.32 None of the tumor samples or cell lines evaluated had evidence of insertions or deletions in exon 11 or exon 17 of either gene. Although it is possible that there are point mutations in the juxtamembrane and catalytic domains that were not detected by this screening process, dysregulation of Kit or Flt3 through deletions and insertions in these regions is extremely unlikely to occur in canine histiocytic sarcoma.

As the known mutations in Met occur over the entire kinase domain, we elected to directly sequence most of this domain including exons 16 through 19. To facilitate this process, primers were designed on the basis of the corresponding introns because gDNA was used and the intervening introns were prohibitively long for PCR procedures. The PCR products for exons 18 through 20 were directly sequenced from the 4 histiocytic sarcoma lines and 34 representative histiocytic sarcoma tumor samples because most mutations in Met are known to occur in these coding regions. No evidence of mutation was found in any of the exons analyzed, although a polymorphism at nucleotide 3711 (C to T, amino acid 1237) was identified. However, this did not change the amino acid sequence. The PCR products for exons 16 to 17 were also directly sequenced from the 4 histiocytic sarcoma lines and 23 representative histiocytic sarcoma tumor samples because rare mutations are found in these coding regions. Again, no mutations were identified.

Therefore, our data suggest that mutations in kinase domain of Met are unlikely to occur in canine histiocytic sarcoma.

Inhibition of Kit, Flt3, and Met signal transduction in histiocytic sarcoma cell lines—Although there was no obvious evidence of mutations in Kit, Flt3, and Met, it was still possible that these signal transduction pathways were active secondary to autocrine stimulation through coexpression of ligand or through point mutations that were not detected in the initial screenings. To test this possibility, the histiocytic tumor cell lines were treated with 2 inhibitors of these pathways. First, we used the previously described multitargeted kinase inhibitor SU11654 that blocks phosphorylation of several split-kinase RTKs, including Kit, Flt3, PDGFR, and VEGFR. It is known that SU11654 inhibits proliferation of murine, canine, and human
tumor cell lines that carry mutations in Kit and Flt3. Additionally, many of those lines underwent apoptosis after 2 to 3 days of culture in the presence of SU11654. In the present study, there was no evidence of apoptosis in any of the histiocytic sarcoma lines evaluated, even when doses far above the known relevant concentrations were used (Figure 3). We then investigated whether SU11654 was capable of slowing or inhibiting cell proliferation. There was no effect on cell numbers after 72 hours of culture in the presence of SU11654 (Figure 4). These experiments suggest that Kit/SCF and Flt3/Flt3L signal transduction are not essential for survival and proliferation of histiocytic sarcoma cells.

To evaluate the ability of Met inhibition to affect cell proliferation, the Met inhibitor PHA665752 was used in a similar assay. In this setting, HGF was added to the tissue culture medium to promote Met signal transduction and potentially induce cycling in the tumor cells. There was no evidence that HGF had a biological effect on the histiocytic sarcoma cells, and PHA665752 did not inhibit cell proliferation (Figure 4). Therefore, our data suggest that Met/HGF signaling is unlikely to play a role in the survival and proliferation of histiocytic sarcoma.

Discussion
Receptor tyrosine kinase dysregulation is now known to play an important role in a variety of human cancers and has recently been identified in naturally occurring tumors in dogs and cats. Identification and characterization of kinase dysregulation are important because these abnormal pathways can be targeted for therapeutic intervention. Indeed, this approach has already proven successful for the treatment of chronic myelogenous leukemias and primary lung cancer in humans by use of drugs such as imatinib mesylate and gefitinib, as well as for treatment of mast cell tumors in dogs via administration of SU11654.

However, application of such targeted agents requires careful molecular characterization of particular tumors because simple expression of a specific kinase does not translate into dysfunction of that kinase. This was proven to be the case in human primary lung cancer; nearly all affected individuals overexpressed EGFR, but only those persons whose tumors carried EGFR mutations responded to treatment.

The purpose of the present study was to evaluate kinase dysregulation in canine histiocytic sarcoma cell lines because this type of tumor is extremely aggressive and there is currently no effective treatment, resulting in death of all affected dogs. Identification of mutated kinases may therefore provide an opportunity for the development of novel agents to treat this disease. For several reasons, we chose to examine 3 particular pathways: Kit/SCF, Flt3/Flt3L, and Met/HGF. Both Kit and Flt3 signaling are important in normal dendritic cell...
Aberrant Met signaling has been identified in a variety of human cancers, and overexpression of this RTK is often associated with a poor long-term prognosis. More importantly, mice expressing Met that has particular mutations in the kinase domain develop histiocytic sarcoma as well as other tumors such as lymphoma and hemangiosarcoma. Our group has previously evaluated the effects of a novel Met inhibitor and shown that this inhibitor was capable of blocking HGF-induced cell cycling as well as migration and scattering of tumor cells in vitro. Several small molecule inhibitors of Met are presently being investigated in clinical trials, thereby providing a potential opportunity for future use in dogs.

To begin evaluating these pathways in histiocytic sarcomas, it was first necessary to develop appropriate reagents. Our group had previously cloned canine Kit, Met, and HGF, and sequences for canine SCF and Flt3L were present in GenBank. We therefore cloned canine Flt3 using a canine myelogenous leukemia cell line as a template. As expected, canine Flt3 had high homology with both human and mouse proteins (90% and 80%, respectively). The sequence was then used to search the canine genome database, and Flt3 was located on canine chromosome 25 (CanFam1.0). These canine sequences were used to generate appropriate PCR primers, and 4 histiocytic sarcoma tumor cell lines were screened for expression of the RTKs and their ligands. All tumor cell lines expressed mRNA for Kit/SCF, Flt3/Flt3L, and Met, and only 1 cell line did not express mRNA for HGF. Protein expression of Kit was confirmed via flow cytometry; appropriate reagents for assessment of canine Flt3 and Met expression are not currently available.

In the present study, histiocytic sarcoma cell lines as well as tumors were evaluated for evidence of mutation in Kit, Flt3, and Met. For the first 2 RTKs, we focused on regions of the gene known to commonly possess activating mutations: the juxtamembrane domain and the catalytic domain. With respect to the juxtamembrane domain, these mutations usually consist of deletions or insertions, whereas point mutations most commonly occur in the catalytic domain and involve codon 814. To facilitate screening of the cell lines and tumor specimens, a previously described technique involving fluorescent PAGE that is capable of identifying PCR products that differ in size by as little as 1 to 2 bp was used. As previously discussed, none of the samples analyzed had evidence of insertions or deletions in exon 11 (encoding the juxtamembrane domain) of Kit or Flt3. Moreover, insertions or deletions in exon 18 (catalytic domain) of either RTK were not detected. Although it was possible that an undetected point mutation in this region may have resulted in constitutive activation, the lack of response to SU11654 even at extremely high doses indicated that such mutations are not likely to be driving cell survival and proliferation in the histiocytic sarcoma cells. Given that SU11654 also effectively inhibited VEGFR and PDGFR signal transduction, it is probable that dysfunction of these pathways is also absent in histiocytic sarcoma. Overall, these data suggest that the split-kinase RTK family is unlikely to be involved in malignant transformation of dendritic cells and, thus, does not serve as a relevant target for therapeutic intervention.

As previously stated, Met mutation is known to result in the development of histiocytic sarcoma in mouse mod-
els. Given the potential link between this RTK and malignant transformation of dendritic cells, we decided to evaluate canine histiocytic sarcoma cells for evidence of Met dysregulation. The expression of mRNA for Met was detected in the histiocytic sarcoma cell lines; 3 of the 4 lines also expressed mRNA for HGF. The cell lines and tumor specimens were also evaluated for mutation in Met. Unlike Kit and Flt3, mutations in Met occur primarily in the kinase domain; the mutations are not localized to a particular codon but occur throughout this domain spanning exons 16 to 20. It was therefore necessary to develop primers that would enable direct sequencing of each exon. Given the enormity of this task, we elected to sequence exons 16 to 20 from all of the cell lines and only 20 of the tumors, as this would likely provide a representative sample from which to determine whether mutations occur with any regular frequency. No mutations were identified in the kinase domain of Met in any of the samples evaluated. The absence of dysfunction was further supported by a lack of response to inhibition of Met signaling with PHA66752. Therefore, as with Kit and Flt3, dysregulation of Met is considered unlikely to play an important role in malignant transformation of canine dendritic cells.

Although the present study did not reveal any evidence of Kit, Flt3, or Met dysregulation in canine histiocytic sarcoma cells, it did provide important information with respect to the application of targeted agents to treat this disease. As endeavors in human oncology have revealed, a comprehensive understanding of the molecular biology of individual cancers is necessary for the successful application of targeted inhibitors. This was clearly shown to be the case for human lung cancer, in which most tumors overexpressed EGFR, but only 10% to 20% responded to EGFR inhibition as a result of mutations in EGFR that led to inappropriate signal transduction.57 Our data suggest that dysregulation of Kit, Flt3, and Met is unlikely to play an important role in canine histiocytic sarcomas; therefore, treatments intended to block these pathways will probably yield little therapeutic benefit in affected dogs. As such, the effective use of targeted agents to treat histiocytic sarcoma in dogs will likely require a more detailed characterization of the molecular biology of this tumor type.

References


## Appendix

### Primers for Flt3 cloning.

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