Expression of receptor tyrosine kinases VEGFR-1 (FLT-1), VEGFR-2 (KDR), EGFR-1, PDGFRα and c-Met in canine primary brain tumours

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Abstract
Inhibition of tumour growth and angiogenesis by targeting key growth factor receptors is a promising therapeutic strategy for central nervous system tumours. Characterization of these growth factor receptors in canine primary brain tumours has not been done. Using quantitative real-time TaqMan polymerase chain reaction (PCR), we evaluated the expression of messenger RNA (mRNA) for five tyrosine kinase growth factor receptors (vascular endothelial growth factor receptor [VEGFR]-1, VEGFR-2, endothelial growth factor receptor [EGFR]-1, platelet-derived growth factor receptor α [PDGFRα], and c-Met) relative to normal cerebral cortex in 66 spontaneous canine primary brain tumours. Increased expression of VEGFR-1 and VEGFR-2 mRNA was greatest in grade IV astrocytomas (glioblastoma multiforme) and grade III (anaplastic) oligodendrogliomas. EGFR-1 mRNA expression was more consistently increased than the other receptors in all tumour types, while increased PDGFRα mRNA expression was mostly restricted to oligodendrogliomas. The similarities in increased expression of these tyrosine kinase growth factor receptors in these canine tumours, as compared to data from their human counterparts, suggest that common molecular mechanisms may be present.

Introduction
Molecular characterization of human primary brain tumours has contributed significantly to a better understanding of tumour biology, development of novel targeted therapies, and the ability to predict clinical progression and response to therapy. All cells contain an integrated network of regulatory pathways that allow diverse cellular events to be controlled via external factors such as peptide growth factors. Activation of these pathways, following the binding of specific growth factors to appropriate receptors, is mediated by phosphorylation–dephosphorylation of protein tyrosine kinases present within the intracellular portion of the receptor. Increased expression of peptide growth factors and their associated tyrosine kinase receptors has been implicated in human brain
tumour proliferation, transformation and angiogenesis. Vascular endothelial growth factor (VEGF) and its major receptors VEGF receptor-1 (VEGFR-1/FLT-1) and VEGF receptor-2 (VEGFR-2/KDR) play a major role in the regulation of tumour angiogenesis, vasculogenesis and vascular permeability in human meningiomas and glial tumours. Increased expression of other growth factors and/or their associated receptors including epidermal growth factor, platelet-derived growth factor (PDGF) and hepatocyte growth factor/scatter factor and its receptor c-Met has also been implicated in angiogenesis and tumour progression in human meningiomas and glial tumours.

In domestic animal species, tumours of the central nervous system occur most frequently in dogs. Meningiomas and glial cell tumours (astrocytoma, oligodendroglioma and mixed glioma) are the most commonly reported tumour types, with glial tumours being especially prevalent in brachycephalic breeds such as the Boxer and Boston terrier. The incidence of primary brain tumours in dogs, approximately 14.5 per 100,000 or 1–3% of all primary neoplasia recorded at necropsy, is similar to that in humans, and their biological behaviour, clinical imaging and histological characteristics have many similarities to their human tumour counterparts. However, minimal data are available on the expression of growth factors and their receptors in canine brain tumours, other than the immunohistochemical demonstration of epidermal growth factor receptor (EGFR) and VEGF expression in astrocytic tumours. In this study, we measured the expression of the major growth factor receptors VEGFR-1 (FLT-1), VEGFR-2 (KDR), EGFR, PDGF receptor α (PDGFRα) and c-Met in spontaneous canine meningiomas, astrocytomas and oligodendrogliomas relative to normal canine cerebral cortex, using quantitative real-time TaqMan polymerase chain reaction (PCR) and correlated the expression with histological tumour type and grade.

Materials and methods

Tissue samples

All tumour tissues were obtained from clinical cases presented to the Veterinary Medical Teaching Hospital, University of California, Davis. Tissues were obtained from surgical biopsy/resections, at necropsy, or from archival paraffin-embedded material. All surgical and necropsy samples were snap frozen and stored in liquid nitrogen. Surgical samples were frozen immediately following excision. Necropsy samples were frozen within 30 min of euthanasia. Whenever frozen tissue was stored, adjacent tumour tissue samples were processed for routine paraffin embedding and histology to provide a microscopic diagnosis. Samples from normal cerebral cortex were collected as controls from both necropsy and archival paraffin-embedded material.

Tumour grading

All tumours were classified and graded by board-certified pathologists (R. J. H., A. W. B.) essentially according to the latest amended criteria of the World Health Organization classification of primary tumours of the human nervous system. Specifically, meningiomas were graded as grade I, grade II (atypical) or grade III (malignant). Oligodendrogliomas were graded as either grade II or grade III (anaplastic). Astrocytomas were graded as grades II, III or IV (glioblastoma multiforme, GBM).

Canine receptor sequences

Canine complementary DNA (cDNA) sequence data for VEGFR-1 (FLT-1) and VEGFR-2 (KDR) were kindly provided by Dr Rolf Jaussi (Institute of Medical Radiobiology of the University of Zurich and the Paul Scherrer Institute, Switzerland). Canine cDNA sequence for c-Met was kindly provided by Dr Cheryl London (The Ohio State University College of Veterinary Medicine). The cDNA encoding canine EGFR-1 sequence spanning exons 15–20 (GenBank accession AY527212) was obtained by PCR from normal canine brain and liver cDNA using primers derived from rat EGFR-1 sequence (GenBank accession NM031507). The cDNA encoding canine PDGFRα sequence spanning exons 11–18 (GenBank accession AY525124) was obtained by PCR from normal canine brain and liver cDNA using primers...
derived from human and murine PDGFRα sequence (GenBank accessions D50007 and NM 011058).

RNA extraction/cDNA preparation

Total RNA was extracted from 30 μm sections of formalin-fixed, paraffin-embedded tissue and reverse transcribed into cDNA as previously described15. The cDNA was analyzed immediately or stored at −20 °C until use. For nucleic acid extraction from snap-frozen surgical and necropsy tissues, 20–25 mg of samples were transferred frozen into 96 well plates containing two grinding beads (4 mm in diameter; SpexCertiprep, Metuchen, NJ, USA), immediately homogenized (GenoGrinder2000; SpexCertiprep) and total RNA extracted from the tissue lysates using a 6700 automated nucleic acid workstation (Applied Biosystems, Foster City, CA, USA).

Real-time TaqMan PCR

Expression of FLT-1, KDR, EGFR-1, PDGFRα and c-Met messenger RNA (mRNA) in brain tumour samples relative to normal brain was determined using real-time TaqMan PCR.

TaqMan PCR systems for canine housekeeping genes glyceraldehyde-3-phosphate dehydrogenase (GAPDH), ribosomal protein L13A, glycosyltransferase (HPRT1), and glucuronidase beta (GUSB), were designed and validated as previously described16. Target gene systems for VEGFR-1, VEGFR-2, EGFR-1, PDGFRα and c-Met were designed based on canine sequences described above using Primer Express software (Applied Biosystems) to standardize reaction conditions and cycling requirements (Table 1). The internal probe was labelled at the 5′ end with the reporter dye FAM (6-carboxyfluorescein) and at the 3′ end with the quencher dye TAMRA (6-carboxytetramethylrhodamine). To allow discrimination between cDNA and genomic DNA, either one of the PCR primers or the TaqMan probe was placed over an exon–exon junction. The PCR reactions contained 400 nM of each primer, 80 nM of the TaqMan probe and commercially available PCR mastermix (TaqMan Universal PCR Mastermix; Applied...

<table>
<thead>
<tr>
<th>Target</th>
<th>Forward primer sequence (5'→3')</th>
<th>Reverse primer sequence (5'→3')</th>
<th>Probe sequence (5'→3')</th>
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<td>CACTGCCCAGATCTTTAATTTGCTT</td>
<td>AGTCTTATTATTACTCGGACTTTTCCTGTGGCTGAA</td>
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Biosystems) and 5 μL of the diluted cDNA sample in a final volume of 12 μL. The samples were placed in 96 well plates and amplified in a combined thermocycler/fluorometer (ABI PRISM 7700 SDS; Applied Biosystems). Amplification conditions were 2 min at 50 °C and 10 min at 95 °C, followed by 40 cycles of 15 s at 95 °C and 60 s at 60 °C. Final quantitation was done using the comparative Ct method and was reported as relative transcription or the n-fold difference relative to a calibrator (mean value for individual normal cerebral cortex samples). Ct values for GAPDH for each tumour group were averaged and compared groupwise. Individual samples that showed significantly lower Ct values for GAPDH (cut-off 3 Ct values weaker than average) were considered low quality cDNA samples and eliminated from the analysis.

Statistical analysis

Data were divided into nominal categories of tumour type, and within these types, they were divided into ordinal categories of grade. Kruskal-Wallis one way analysis of variance was used to compare the variation in expression of canine receptor tyrosine kinase mRNA among tumour types. When significant differences were evident, Mann–Whitney tests were used for pairwise comparisons. Within tumour type, evidence of a trend in expression across ordinal grades was evaluated using the Jonckheere–Terpstra test. Side-by-side box plots were used to nonparametrically summarize the distribution of the dependent variables. Statistical significance was defined as P < 0.05.

Results

A total of 15 samples of normal cerebral cortex (4 frozen and 11 paraffin-embedded) and 66 tumour samples (13 frozen and 53 paraffin-embedded) were analyzed. Of these samples, there were 23 meningiomas (9 grade I, 13 atypical and 1 malignant), 23 astrocytomas (7 grade II, 8 grade III and 8 GBM) and 20 oligodendrogliomas (2 grade II and 18 grade III). For all genes analyzed, the averages of normalized values and the standard deviations for both frozen and paraffin-embedded samples were not significantly different.

VEGFR-1 (FLT-1) expression

Product was amplified from 10 meningiomas, 18 astrocytomas and 20 oligodendrogliomas (Fig. 1A). Increased expression was seen predominantly in glial tumours, with the highest expression in GBMs and oligodendrogliomas. Expression in high-grade (III) oligodendrogliomas was significantly increased relative to low-grade astrocytomas (II) (P < 0.0002) and meningiomas (P < 0.0001). Expression in GBMs was significantly greater than meningiomas (P < 0.00072), and there was a significant trend of increasing expression with increasing grade of astrocytic tumour (grade II, grade III and GBM) (P < 0.010).

VEGFR-2 (KDR) expression

Product was amplified from 19 meningiomas, 20 astrocytomas and 20 oligodendrogliomas (Fig. 1B). As with VEGFR-1, increased expression was seen predominantly in glial tumours. Expression in GBMs and oligodendrogliomas was significantly greater than in meningiomas of all grades (P < 0.045 and P < 0.032, respectively). Expression in high-grade meningiomas (atypical/malignant) was significantly greater than in grade I meningiomas (P < 0.00024).

EGFR-1 expression

Product was amplified from 17 meningiomas, 19 astrocytomas and 20 oligodendrogliomas (Fig. 1C). Increased expression of EGFR-1 was found in all tumour types; however, the majority of grade I meningiomas had low expression relative to other tumour types and high-grade meningiomas. Greatest expression was seen most consistently in the high-grade gliomas (GBMs and oligodendrogliomas). Expression in oligodendrogliomas was significantly greater than in grade I meningiomas (P < 0.0001).

PDGFRα expression

Product was amplified from 10 meningiomas, 11 astrocytomas and 20 oligodendrogliomas (Fig. 1D). Increased expression was most often found
in oligodendrogliomas. Expression in oligodendrogliomas was significantly greater than in GBMs, grade II astrocytomas and grade I meningiomas ($P < 0.0004$, $P = 0.0003$ and $P < 0.0001$, respectively).

c-Met expression

Product was amplified from 12 meningiomas, 13 astrocytomas and 19 oligodendrogliomas (Fig. 1E). Trends of increasing expression with increasing tumour grade occurred in both meningiomas and astrocytic tumours, although not a statistically significant difference. The greatest increases in expression occurred in oligodendrogliomas.

Discussion

Increased expression of both mRNA and protein has been demonstrated previously for VEGFR$^{3-5}$, PDGFR$^{14-16,37}$, EGFR$^{8,9,13}$ and c-Met$^{17}$ in human astrocytomas, oligodendrogliomas and...
Based on these data, experimental therapies targeting defined growth factors and their tyrosine kinase receptors have progressed into human clinical trials. Effective translation of these therapeutic strategies into canine brain tumours will require identification of similar molecular abnormalities. Little information is available relating to expression of growth factor receptors in spontaneous canine cancer of any type. Increased expression of VEGFR-1 has been described in canine mammary tumours, melanomas, fibrosarcoma, mastocytoma and pancreatic carcinoma, while reports of increased EGFR and c-Met expression have been limited to canine mammary tumours and osteosarcomas, respectively. The data presented suggest that altered tyrosine kinase growth factor pathways including VEGFR-1, VEGFR-2, EGFR, PDGFRα and c-Met may be present in canine meningiomas and glial tumours, similar to those in their human tumour counterparts.

**VEGFR expression**

Increased expression of VEGFR-2 was found in human meningiomas, astrocytomas and oligodendroglomas, with greatest expression in high-grade glial tumours. A similar pattern of expression has been reported for VEGFR-1; however, the data are less consistent. The pattern of expression of VEGFR-2 mRNA in canine tumours was very similar to those in their human counterparts, with the greatest expression seen in grade III oligodendroglomas and GBMs and lower expression in meningiomas. VEGFR-1 expression was also greatest in high-grade gliomas similar to equivalent human tumours. Expression of VEGF receptors is rarely seen in normal brain vasculature and is predominantly located in endothelial cells within tumour vasculature. However, expression has also been reported in non-vascular elements of tumour tissue from both human brain tumours and canine tumours. Expression of VEGF receptors is induced during tumour progression and probably plays a major role in tumour angiogenesis. Histological assessment of microvascular density was not done in this study; however, the greatest expression of VEGF receptors was seen in high-grade gliomas, where microvascular proliferation is a specific criterion for tumour grading. The significance of decreased VEGFR-1 mRNA expression in some canine tumour samples (particularly meningiomas) is unknown; however, it has been suggested that the function of VEGFR-1 may be inhibitory via regulation of VEGF bioavailability following ligand binding to alternatively spliced soluble receptors. Downregulation of VEGFR-1 may provide a biological advantage for tumour growth in this situation.

**EGFR expression**

In human glial tumours, increased EGFR-1 expression was seen predominantly in high-grade astrocytomas, usually as a result of gene amplification and/or gene rearrangement, and in oligodendroglomas. Increased expression was seen in meningiomas, although at a lower level, with no consistent association between increased EGFR-1 expression and meningioma grade or histological subtype. In this study, increased EGFR-1 expression was also present in all canine tumour types and grades and was a more consistent finding than for the other receptors (VEGFR, PDGFRα and c-Met) where increased expression was most typically seen in high-grade gliomas. Increased EGFR-1 expression has been reported in a small number of low-grade human astrocytomas, and this was also found in canine low-grade astrocytomas. Similar findings were reported for EGFR-1 by Stoica et al., where three of six canine astrocytomas with positive immunostaining for EGFR-1 were low-grade (II/III) astrocytomas.

**PDGFR expression**

Increased expression of PDGFRα, PDGFRβ and PDGF A and B has been demonstrated in both high- and low-grade human astrocytomas, as well as in oligodendroglomas. In human meningiomas, only PDGFRβ expression is increased. In agreement with these findings, increased PDGFRα expression was not seen in any of the canine meningioma samples. The pattern of expression of PDGFRα in canine tumours was different from that seen with the other growth factor.
receptors, in that increased expression was seen predominantly in oligodendrogliomas. The minimally increased expression of PDGFRα in these canine astrocytomas may indicate that either PDGFRα does not have a major role in canine astrocytomas compared to their human counterparts or signalling may be occurring predominantly via the β receptor.

c-Met expression

Increased expression of c-Met has been reported in human astrocytomas, particularly in high-grade tumours17–19. Limited data exists regarding meningiomas and oligodendrogliomas; however, c-Met has been demonstrated immunohistochemically in meningiomas in one study17. Increased expression of c-Met was not found in one study looking at ungraded oligodendrogliomas19.

Expression of c-Met was relatively low in most of the canine tumour samples in this study compared to the increased expression seen with other growth factor tyrosine kinase receptors. Consistent increased expression was only seen in the grade III canine oligodendroglial tumours. The majority of human oligodendrogliomas are grade II tumours in contrast to the majority of grade III oligodendrogliomas in this study. This difference may explain the increased c-Met expression seen in oligodendrogliomas in this study compared to human oligodendrogliomas19.

The relatively small sample size and selective sampling from individual tumours limit the conclusions that may be drawn from the data presented, particularly with respect to individual tumour samples. However, the results of this study provide evidence that increased expression of several major tyrosine kinase growth factor receptors is a common finding in canine primary brain tumours, particularly in high-grade gliomas.

Canine primary tumours have striking similarities to their human counterparts in terms of biological behaviour, imaging and histological characteristics. Increased expression of receptor tyrosine kinases in canine brain tumours suggests that similarities may also be present at the molecular level. This finding validates the use of growth-factor-targeted therapies as a rational treatment strategy in dogs and supports the use of spontaneous canine primary brain tumours as a model system for human disease.

Acknowledgments

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