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Immunohistochemical evaluation of tissue factor, fibrin/fibrinogen and D-dimers in canine gliomas

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ABSTRACT

In human gliomas, tissue factor (TF) is overexpressed, associated with the grade of malignancy and influences tumour biology. Intra-tumoural fibrin/fibrinogen deposition and activation of the fibrinolytic system also play a role in tumour cell proliferation and angiogenesis. The first aim of the present study was to investigate TF expression and the presence of fibrin/fibrinogen and D-dimers in canine glioma biopsies, graded according to the World Health Organization (WHO) classification of tumours of the central nervous system. The second aim was to investigate the occurrence of intravascular thrombosis (IVT) in canine gliomas, as a potential histological marker of glioma type or grade of malignancy.

An immunohistochemical study using antibodies against TF, fibrin/fibrinogen and D-dimers was performed with 24 glioma samples, including 15 oligodendrogliomas, 6 astrocytomas and 3 mixed gliomas. Immunohistochemical data were statistically analysed to determine whether there was any relationship between glioma type and grade of malignancy. All gliomas were moderate to strongly positive for TF and the staining score was significantly higher ($P = 0.04$) in high-grade (III or IV) than in low-grade (II) gliomas. Intra-tumoural fibrin/fibrinogen deposition was detected in all tumour biopsies assessed, and D-dimers were detected in 17/24 gliomas. IVT was a frequent finding, but was not linked to a specific glioma type or malignancy grade. TF expression, fibrin/fibrinogen deposition, extravascular fibrinolytic system activation and IVT occur in canine gliomas. Canine glioma might be a suitable model for studying coagulation and fibrinolysis as potential therapeutic targets for human gliomas.

Introduction

The role of the haemostatic system in cancer biology has long been recognised, and several studies have demonstrated that some of these components influence angiogenesis, facilitating tumour growth and metastasis (Langer and Bokemeyer, 2012). Research has focused on the extravascular activation of the coagulation and fibrinolytic systems in order to elucidate their role beyond the intravascular compartment. Thus, the biological actions of some components of the haemostatic system are currently considered to be not only associated with blood clotting but also as pleiotropic proteins that are important for tumour progression (Wojtukiewicz et al., 2001; Langer and Bokemeyer, 2012). Fibrin/fibrinogen and tissue factor (TF; the initiator of the extrinsic coagulation cascade) are currently subject to investigation, to determine whether an activated coagulation cascade in the tumour microenvironment might be an effective therapeutic target (Liu et al., 2011).

TF expression in human solid tumours was first reported some time ago (Callander et al., 1992). More recently it was shown that TF triggers cellular signalling pathways that can induce tumour growth, angiogenesis and metastasis (Mueller and Ruf, 1998; Hjortoe et al., 2004; Yu et al., 2005; Versteeg et al., 2007). TF is overexpressed in human gliomas and the degree of TF expression is associated with the histological grade of malignancy and vascularity (Hamada et al., 1996; Guan et al., 2002; Thaler et al., 2013). TF expression has also been demonstrated in different canine tumour cell lines, particularly those derived from epithelial tumours (Stokol et al., 2011), but there is little information about TF expression in canine gliomas.

Extravascular activation of the coagulation cascade, within the tumour microenvironment, leads to fibrin/fibrinogen deposition, which provides a provisional extracellular matrix to facilitate angiogenesis, binding of growth factors (such as vascular endothelial growth factor, VEGF) and promoting cell adhesion, proliferation and migration. Fibrin/fibrinogen might also act as a barrier between the tumour and host that could interfere with anti-tumour immune responses (Dvorak et al., 1983; Simpson-Haidaris and Rybarczyk, 2001; Staton et al., 2003). Fibrin/fibrinogen deposition has been demonstrated in the majority of human tumour types, including primary

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and metastatic brain neoplasms (Bardos et al., 1996). There are few reports describing fibrin/fibrinogen deposition in canine tumours (McEvoy et al., 1996; Golombiewski et al., 1997), but evidence is lacking regarding expression in central nervous system (CNS) tumours.

The components of the fibrinolytic system and fibrin degradation-related fragments also play a role in tumour angiogenesis and are critical for tumour progression (Bootle-Wilbraham et al., 2001; Wojtukiewicz et al., 2001; Staton et al., 2003). However, the relationship between fibrinolysis and angiogenesis is not fully understood because of the coexistence of both pro- and anti-angiogenic properties of some of their components (Wojtukiewicz et al., 2001). For example, fibrin fragment E is a potent angiogenic factor that enhances the effect of VEGF (Bootle-Wilbraham et al., 2001), whereas cleavage of plasminogen releases proteolytic fragments, such as angiostatin, which are potent inhibitors of angiogenesis (Wojtukiewicz et al., 2001). Although the influence of D-dimers on angiogenesis is unknown, these represent specific degradation products of cross-linked fibrin and can be used as specific markers of fibrinolysis. A recent study has demonstrated that there is fibrinolytic activity in the cerebrospinal fluid of dogs affected with glioma (de la Fuente et al., 2012).

The aim of the present study was to use immunohistochemistry to identify and characterise TF expression, as well as fibrin/fibrinogen and D-dimer deposition in canine gliomas. Since intravascular thrombosis (IVT) has been proposed as the underlying mechanism that leads to hypoxia-induced tumour progression in human gliomas (Tehrani et al., 2008), the secondary aim was to investigate the occurrence of IVT in canine gliomas, as a potential histological marker of glioma type or grade of malignancy.

Materials and methods

Study population

The veterinary neuropathology database of the Universitat Autònoma de Barcelona was searched to identify dogs affected with brain neoplasia between 2001 and 2013. Inclusion criteria for the study were that a histopathological diagnosis of brain glioma had been made, and that there was archival tissue available that was suitable for immunohistochemical studies. All samples were reviewed by an ECVP Board-certified pathologist (MP), who classified and graded all cases according to the World Health Organization (WHO) classification of CNS tumours (Louis et al., 2007).

Oligodendroglial tumours were classified as grade II (oligodendroglioma) or grade III (anaplastic oligodendroglioma), and astrocytomas were classified as grade II (diffuse astrocytoma), grade III (anaplastic astrocytoma) or grade IV (glioblastoma). Mixed glial tumours were graded as either II or III. Gliomatosis cerebri was considered a grade III astrocytic tumour (Stoica et al., 2011).

Immunohistochemistry

Formalin-fixed, paraffin-embedded tissue sections (3 μm) were dewaxed with xylene and rehydrated in descending concentrations of ethanol. For antigen retrieval, tissue sections were boiled in bain-marie (96–98 °C) for 20 min with 0.01 M citrate buffer (pH 6.0) (TF, D-dimer) or treated with 0.1% trypsin in distilled water for 20 min (fibrin/fibrinogen). Endogenous peroxidase activity was suppressed with 3% H_2O_2 for 40 min. Slides were blocked with goat serum for 1 h. TF immunolabelling was performed using a monoclonal rabbit anti-human antibody (ab151748, Abcam) at a dilution of 1:400. The positive control tissue consisted of sections of canine mammary gland carcinoma, a neoplasia with high TF expression (Stokol et al., 2011).

Fibrin/fibrinogen immunoreactivity was assessed using a polyclonal rabbit anti-human fibrin/fibrinogen antibody (A0080, Dako) at a dilution of 1:10,000 as described previously (Cotovio et al., 2007). This antibody reacts with fibrin, fibrinogen and fibrinogen fragments D and E. To verify immunoreactivity with canine fibrin/fibrinogen, sections from a brain with intra-parenchymal haemorrhages were assessed as a positive control. D-dimer immunolabelling was performed with a monoclonal mouse anti-D-dimer antibody (ABS 015-22-02, Thermo Scientific) as described previously, but at a 1:20,000 dilution (Carretón et al., 2013). Immunohistochemistry for TF and fibrin/fibrinogen was carried out using an EnVision+ System-HRP (DAB) Rabbit Kit (Dako), with an EnVision+ System-HRP (DAB) Mouse Kit (Dako) used for D-dimers. The chromogen substrate used was 3, 3′ diaminobenzidine (Dako) and counterstaining was performed with haematoxylin (Merck). Negative control tissue sections from the same specimens were identically processed, replacing the specific primary antibody with an isotype-control IgG of the same species and concentration as the primary antibody.

The intensity of TF staining was graded, based on the estimated proportion of the tumour cell population showing positive staining for TF (Hamada et al., 1996): negative (score of 0: no positive cells detected); weakly positive (score of 1: <50% positive tumour cells); moderately positive (score of 2: ≥50% positive tumour cells with weak intensity); strongly positive (score of 3: ≥50% positive tumour cells with strong intensity). Fibrin/fibrinogen and D-dimer immunoreactivity were graded using a modification of a scoring system described previously (Cotovio et al., 2007). Briefly, 20 high power fields (HPF; 40× objective lens) were evaluated within each tumour, then fibrin/fibrinogen or D-dimer deposition was graded as either 0: no staining or <10% positive fields; 1: 11–25% positive fields; 2: 26–50% positive fields; 3: 51–75% positive fields; 4: ≥76% positive fields with staining in an almost continuous pattern.

IVT was assessed in canine gliomas, by evaluating the presence of thrombosed vessels within each tumour, classified as one that was occluded by a deposit of D-dimers. The number of thrombosed vessels was counted in 20 HPF in triplicate. The results, expressed as mean and standard deviation of thrombosed vessels per HPF, were compared for each histological type, malignancy grade and TF scores.

Statistical analysis

Data analysis was performed using commercial software (SPPS, version 18.0). Frequencies and percentages were used for qualitative variables and range for age of dogs. Quantitative results for variables not normally distributed are expressed as median and 25th–75th percentiles. For inferential analysis, the chi-square test was used to compare the scores of each stain in different glioma types and malignancy grades. One-way ANOVA with Bonferroni adjustment was used to detect differences between the mean of thrombosed vessels in each glioma type, malignancy grade and TF score. Significance was set at $P < 0.05$ for all tests.

Results

Study population

Twenty-four dogs diagnosed with brain gliomas met the inclusion criteria. Dogs were between 4 and 15 years of age (median 8.5 years). Six dogs had grade II oligodendrogliomas, nine dogs had grade III oligodendrogliomas, one dog had a grade II astrocytoma, four dogs had grade III astrocytomas (including one dog with gliomatosis cerebri), one dog had a grade IV astrocytoma (glioblastoma), one dog had grade II mixed glioma and two dogs had grade III mixed gliomas.

Tissue factor in canine gliomas

Immunoreactivity to TF was detected in all 24 glioma specimens, and was moderate (score 2; 14/24) or strongly positive (score 3; 10/24) as shown in Table 1. The pattern of TF expression was diffuse and homogeneous in 6/24 specimens (two grade II oligodendrogliomas, two grade III oligodendrogliomas, one grade IV astrocytoma and one grade II mixed glioma). Most gliomas showed a heterogeneous pattern of TF immunostaining, characterised by a lack of or low expression of TF in the periphery of the tumour, and a progressive increase of staining close to the tumour core.

Expression of TF was marked in the tumour cell population surrounding the microvasculature and around necrotic and haemorrhagic areas (Figs. 1A and B). Scattered clusters of tumour cells with angiocentric distribution and strong TF immunolabelling were visible in all specimens, even in gliomas with a moderate staining score (2). At the cellular level, TF immunolabelling was usually stronger at the cell surface than in the cytoplasm of glial cells. No TF immunoreactivity was observed in the adjacent normal brain tissue, except for scattered reactive astrocytes, surrounding the tumour, which showed strong TF expression.

No statistical differences were found in TF scores, comparing different glial tumour histological types (oligodendrogliomas *versus* astrocytomas *versus* mixed gliomas) or between different malignancy grades, within a specific glioma type. However, when all gliomas were grouped according to grade, the TF score was significantly greater for high-grade (III or IV) compared with low-grade (II) gliomas ($P = 0.04$).