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Expression of platelet-derived growth factor BB, erythropoietin and erythropoietin receptor in canine and feline osteosarcoma

F.R.L. Meyer [a], R. Steinborn [b], H. Grausgruber [c], B. Wolfesberger [d], I. Walter [a,*]

[a] Institute of Anatomy, Histology and Embryology, Department of Pathobiology, University of Veterinary Medicine, Veterinaerplatz 1, 1210 Vienna, Austria
[b] Genomics Core Facility, VetCore, University of Veterinary Medicine, Veterinaerplatz 1, 1210 Vienna, Austria
[c] Division of Plant Breeding, University of Natural Resources and Life Sciences, Vienna, Konrad Lorenz-Strasse 24, 3430 Vienna, Austria
[d] Department for Companion Animals and Horses, University of Veterinary Medicine, Veterinaerplatz 1, 1210 Vienna, Austria

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ABSTRACT

The discovery of expression of the erythropoietin receptor (EPO-R) on neoplastic cells has led to concerns about the safety of treating anaemic cancer patients with EPO. In addition to its endocrine function, the receptor may play a role in tumour progression through an autocrine mechanism. In this study, the expression of EPO, EPO-R and platelet-derived growth factor BB (PDGF-BB) was analysed in five feline and 13 canine osteosarcomas using immunohistochemistry (IHC) and reverse transcription polymerase chain reaction (RT-PCR).

EPO expression was positive in all tumours by IHC, but EPO mRNA was only detected in 38% of the canine and 40% of the feline samples. EPO-R was expressed in all samples by quantitative RT-PCR (RT-qPCR) and IHC. EPO-R mRNA was expressed at higher levels in all feline tumours, tumour cell lines, and kidney when compared to canine tissues. PDGF-BB expression was variable by IHC, but mRNA was detected in all samples. To assess the functionality of the EPO-R on tumour cells, the proliferation of canine and feline osteosarcoma cell lines was evaluated after EPO administration using an alamarBlue assay and Ki67 immunostaining. All primary cell lines responded to EPO treatment in at least one of the performed assays, but the effect on proliferation was very low indicating only a weak responsiveness of EPO-R. In conclusion, since EPO and its receptor are expressed by canine and feline osteosarcomas, an autocrine or paracrine tumour progression mechanism cannot be excluded, although in vitro data suggest a minimal role of EPO-R in osteosarcoma cell proliferation.

Introduction

Erythropoietin (EPO) is a small glycoprotein hormone which mostly promotes erythropoiesis, but has also non-haematopoietic, growth-promoting and apoptosis-inhibiting roles (Noguchi et al., 2008; Debeljak et al., 2014; Wan et al., 2014). Recently, it has been shown in a mouse model that angiogenesis in tumours is modulated by inducing EPO production via platelet-derived growth factor BB (PDGF-BB) in tumour stromal cells (Xue et al., 2012). This system is based on a complex paracrine mechanism involving tumour cells, stromal cells and, in particular, tumour blood vessels. In addition, high amounts of circulating EPO have been shown to stimulate proliferation, migration and cell sprouting of endothelial cells (Xue et al., 2012).

In adults, EPO is mainly produced in the kidneys. It binds to the erythropoietin receptor (EPO-R) and activates JAK-STAT-signalling (Noguchi et al., 2008). EPO-R is expressed predominately on erythrocyte progenitors and EPO can be used therapeutically in humans and animals to treat anaemia (Eschbach and Adamson, 1985). Human cancer patients with myelodysplastic disorders are frequently treated with EPO but there is an ongoing discussion about the effect of EPO on tumour cells. Positive effects of EPO for humans (Ludwig et al., 1994; de Campos et al., 1995; Demetri et al., 1998; Mittelman et al., 2001; Wallvik et al., 2002; Jädersten et al., 2008) and animals (Langston et al., 2003) have been reported. However, several clinical studies showed worse survival times and shorter disease-free intervals after EPO treatment in cancer patients (Henke et al., 2003, 2006; Leyland-Jones et al., 2005; Savonije et al., 2005; Bohlius et al., 2009).

The presence of EPO and EPO-R has been demonstrated in various tumours in humans and animals (Acs et al., 2001; Arcasoy et al., 2002; Batra et al., 2003; Sfacteria et al., 2005; Klopfleisch et al., 2012; Rózsás et al., 2013; Reinbothe et al., 2014) and EPO has been shown to promote angiogenesis, vessel maturity, drug resistance, proliferation and tumour progression (Morais et al., 2013; Asano et al., 2015). Others have questioned the functionality of EPO-R on tumour cells (Sinclair et al., 2007) due to its low level of expression (Swift

* Corresponding author. Tel.: +43 1 25077 3406.
E-mail address: Ingrid.Walter@vetmeduni.ac.at (I. Walter).

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et al., 2010). In addition, treatment of EPO-R-expressing cell lines with EPO did not enhance cell proliferation in vitro (Westphal et al., 2002; Farrell and Lee, 2004).

Osteosarcoma is considered to be the most frequent bone tumour in dogs and cats (Quigley and Leedale, 1983) but the metastatic behaviour differs between the two species, with frequent distant metastases in dogs (Spodnick et al., 1992) and a remarkably low metastatic rate in cats (Dimopoulou et al., 2008). To our knowledge, no studies about EPO and its receptor in bone tumours of cats or dogs have been reported. Recently, PDGFs and PDGF receptors (PDGF-R) were found to be overexpressed in canine osteosarcoma (Xue et al., 2012; Maniscalco et al., 2013). Moreover, PDGFs and PDGF-Rs were co-expressed, suggesting that an autocrine and/or paracrine loop could be involved (Xue et al., 2012; Maniscalco et al., 2013). No data about PDGFs exist in feline osteosarcomas.

We hypothesised that the expression pattern of EPO, EPO-R and PDGF-BB in canine and feline osteosarcoma cells differs between the two species and that this may influence tumour growth, angiogenesis and the different metastatic behaviour in the two species.

Materials and methods

Sample collection

Tumour samples from dogs ($n = 13$) and cats ($n = 5$) were collected after therapeutic limb amputation or euthanasia. Control samples (kidney, bone marrow, cerebellum) were obtained from animals euthanased for reasons other than osteosarcoma. Material was collected from surgery (with informed owner's consent) or from cadaveric clinical waste and was conveyed to the Institute of Pathology of the University of Veterinary Medicine. The study was approved by the Ethical and Animal Welfare Committee of the University of Veterinary Medicine (15 December 2014) and conducted in accordance with the requirements of the Austrian Act on Animal Experiments.

Aliquots of samples were either fixed in 4% buffered formaldehyde or flash frozen in liquid nitrogen with or without RNA later solution (Life Technologies).

Immunohistochemistry

For immunohistochemistry (IHC), paraffin sections were used and stained with either an anti-EPO, (sc-7956, polyclonal, dilution 1:50; Santa Cruz Biotechnology), an anti-EPO-R (C-20, sc-696, polyclonal, dilution 1:50; Santa Cruz Biotechnology), an anti-EPO-R (M-20 sc-697, polyclonal, dilution 1:75; Santa Cruz Biotechnology) or an anti-PDGF-BB antibody (ab21234, polyclonal, dilution 1:500; Abcam). The evaluation score suggested by Detre et al. (1995) was used. Specificity of the assays was tested by immunoblotting (see Appendix: Supplementary material).

Cell culture

Commercially available canine osteosarcoma cells D-17 (LGC Standards) or primary tumour-derived neoplastic cells (canine: COS_1186w and COS_1189; feline: FOS_1077 and FOS_1140; Lonza) were used. Cells were grown in Dulbecco's modified eagle's medium (DMEM) low glucose with 10% foetal calf serum (FCS, Sigma-Aldrich) and 625 pg Amphotericin B (Bio&Sell), 2 nM glutamine (Biochrom) and 1% Pen/Strep/Fungi Mix (Bio&Sell).

AlamarBlue test

Cells were exposed to different concentrations of EPO and proliferation was determined with the alamarBlue test (Resazurin, Sigma Aldrich) to evaluate the functionality of EPO-R as suggested previously (Levine et al., 2005; LaMontagne et al., 2006; Laugsch et al., 2008). Briefly, 2000 cells/well were seeded in flat bottomed 96-well plates (Sarstedt) and supplemented with 0, 0.001, 0.01, 0.1, 1 or 10 U/mL of alpha erythropoietin (Epoietin, Binocrit 1000 IU/0.5 mL, Sandoz) in 5 μL PBS, or 5 μL PBS alone (control) with or without 10% FCS in the medium. Cells were incubated with EPO for 48 h before 10% alamarBlue was added, and the 570–600 nm ratio was measured on a plate reader (Sunrise, Tecan).

Ki67 immunocytochemistry

For Ki67 immunocytochemical staining, 23,000 (for D-17, FOS_1077 and FOS_1140) or 46,000 cells (for COS_1189 and COS_1186w) were seeded onto round glass cover slides, and supplemented with 0.001, 1 or 10 U/mL alpha erythropoietin (Epoietin, Binocrit, Sandoz) in 5 μL PBS or PBS alone with or without 10% FCS in the medium. After 48 h, cells were fixed in 4% formaldehyde and stained using an anti-Ki67 antibody (clone 7B11, Life Technologies). For the analysis of Ki67 staining, approximately 1000 cells/slide were counted and classified as stained or unstained to generate a percentage of positive cells.

PCR

About 30 mg of fresh frozen or RNAlater (Life Technologies)-preserved tissue or pellets of 10^5–10^6 cultured cells were used for RNA isolation using either the miRNeasy Mini Kit (Qiagen) or the RNeasy Fibrous Tissue Mini Kit (Qiagen). Concentration and the RNA Integrity Number (RIN) were determined with a 2100 Bioanalyzer (Agilent Technologies) (see Appendix: Supplementary material). Using the High-Capacity cDNA Reverse Transcription Kit (Life Technologies), 500 ng RNA/sample was transcribed into cDNA. Feline and canine *EPO* and *PDGF-B* mRNA were detected by qualitative dye-based PCR. Qualitative assessment was performed by melting curve analysis. Canine *EPO* mRNA was measured with a qualitative hydrolysis-probe PCR assay. Quantification of *EPO-R* mRNA was performed by a conventional hydrolysis probe-based format. To compensate for technical variance, data were normalised with the reference genes *OAZ1* (De Jonge et al., 2007; Kwon et al., 2009) and the canine and feline orthologues of human *C12orf43* (Tsai and Breen, 2012).

Statistical evaluation

RT-qPCR data were analysed using REST 2009 (Qiagen). For the Ki67 and alamarBlue assays, significance was assessed by a linear regression model. A detailed description of the statistical methods is provided in the Appendix: Supplementary material.

Results

Immunohistochemistry

Total scores for EPO IHC ranged from 2 to 9 in feline tumours and from 3 to 9 in canine tumours. EPO immunostaining resulted either in a homogenous or heterogeneous pattern within the tumour area. Cytoplasmic and nuclear staining was present (Figs. 1a, b) with additional staining of giant cells and partial staining of endothelial cells within the tumour. In the canine and feline kidney (positive controls), tubules and collecting ducts were stained. Negative controls were negative.

EPO-R IHC resulted in a cytoplasmic staining pattern (total scores ranged from 2 to 9 in both species); a clear membrane staining was not observed (Figs. 1c, d). No differences were found when comparing the C-20 and the M-20 antibodies. All negative controls were negative.

The immunostaining pattern for PDGF-BB was considerably heterogeneous in the osteosarcomas of cats and dogs and total scores ranged from 0 to 9. The staining pattern in canine and feline tumour cells was cytoplasmic (Fig. 1e). In feline osteosarcomas a perivascular distribution of positive cells was partly observed (Fig. 1f). In addition to the tumour cells, giant cells and osteoblasts were positive for PDGF-BB immunostaining in both species. In the positive controls (feline and canine cerebellum), neurons were stained as expected and negative controls were unstained. IHC results are summarised in Table 1.

Table 1
Distribution of immunohistological scores in canine and feline osteosarcomas.

Total IHC score	EPO ($n = 18$)		EPO-R ($n = 18$)		PDGF-BB ($n = 18$)	
	Dog	Cat	Dog	Cat	Dog	Cat
0	0	0	0	0	0	1
1	0	0	0	0	2	0
2	0	1	1	1	5	1
3	2	0	4	0	3	1
4	1	2	1	1	0	0
6	7	0	5	1	3	0
9	3	2	2	2	0	2

EPO, erythropoietin; EPO-R, erythropoietin receptor; PDGF-BB, platelet-derived growth factor BB.