available at www.sciencedirect.com

ScienceDirect

journal homepage: www.ejconline.com

Interrelation of directly measured oxygenation levels, erythropoietin and erythropoietin receptor expression in spontaneous canine tumours

Carla Rohrer Bley[a,*,d], *Melanie Wergin*[a,d], *Malgorzata Roos*[b], *Beat Grenacher*[c], *Barbara Kaser-Hotz*[a]

[a]*Section of Diagnostic Imaging and Radiation Oncology, Vetsuisse Faculty, University of Zurich, Winterhurerstrasse 260, CH-8057 Zurich, Switzerland*
[b]*Biostatistics, ISPM, University of Zurich, CH-8006 Zurich, Switzerland*
[c]*Institute of Veterinary Physiology, Vetsuisse Faculty, CH-8057 Zurich, Switzerland*

ARTICLE INFO

Article history:
Received 9 August 2006
Received in revised form
6 December 2006
Accepted 11 December 2006
Available online 24 January 2007

Keywords:
Tumour hypoxia
Erythropoietin
Erythropoietin receptor

ABSTRACT

The expression of the hypoxia-inducible protein erythropoietin in tumour cells correlates with levels of tumour hypoxia. Our aim was to look for an interrelation of *directly* measured oxygenation levels, the presence of tissue erythropoietin and its receptor. Data of tumour oxygenation status, plasma and tissue erythropoietin and its receptor in a group of spontaneously occurring tumours in 15 dogs were collected. Polarographic tumour oxygen partial pressure measurements were obtained and data were correlated.

Significant positive correlations were found between tissue erythropoietin and the percentages of pO_2 values $\leqslant$10 mmHg. Multivariate analysis revealed no parameters influencing plasma erythropoietin levels.

Our results show that a co-expression of erythropoietin receptor and its ligand in spontaneous canine tumours exists, that the level of hypoxia in tumour cells correlates with the level of tissue erythropoietin and suggest the need to be quantitatively and functionally tested as novel prognostic biological parameters in neoplastic tissues.

1. Introduction

It has been shown that Epo and EpoR are produced and expressed not only by various non-erythroid tissues, but also by human malignancies.[1–3] As a pleiotropic cytokine, Epo on the one hand acts as a haematopoietic growth factor, but it also exerts proangiogenic and tissue-protective effects in other organs. Activation of Epo–EpoR signalling pathways in cancer cells may be followed by modulation of tumour environment such as angiogenesis, increased proliferation and changes in apoptotic ability.[4] Furthermore response to treatment such as chemoradiation may be influenced.

Tumour hypoxia, a factor associated with tumour aggressiveness, treatment resistance and poor prognosis itself,[5–7] seems to be one of the upregulators and modulators of Epo production as a direct result of hypoxia inducible factor-1 activation.[4,8] It has been shown in vivo and in vitro that Epo and EpoR expression can be partially colocalised with tumour tissue hypoxia.[3,9] However, these studies were assessing the

* *Corresponding author:* Tel.: +41 44 635 8487; fax: +41 44 635 8940.
E-mail address: crohrer@vetclinics.unizh.ch (C. Rohrer Bley).
[d] The authors Rohrer Bley and Wergin have equally contributed to this manuscript.
Abbreviations: Epo, erythropoietin; EpoR, erythropoietin receptor; pO_2, partial pressure oxygen tension.
0959-8049/$ - see front matter © 2006 Elsevier Ltd. All rights reserved.
doi:10.1016/j.ejca.2006.12.007

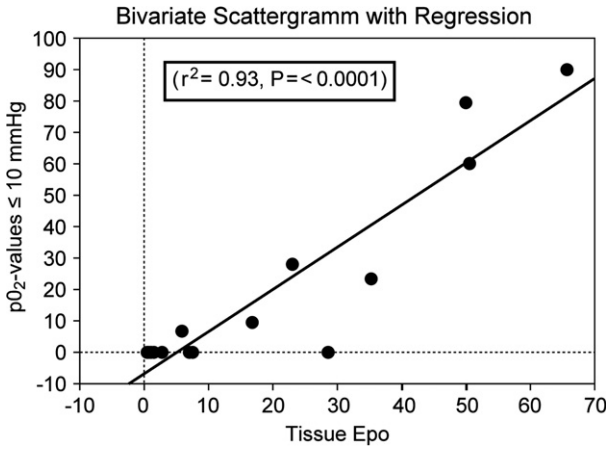

Fig. 1 – Correlations between tissue Epo and the percentages of pO_2 values ⩽10 mmHg: the more values of low oxygen tension are present, the higher is the tissue Epo expression.

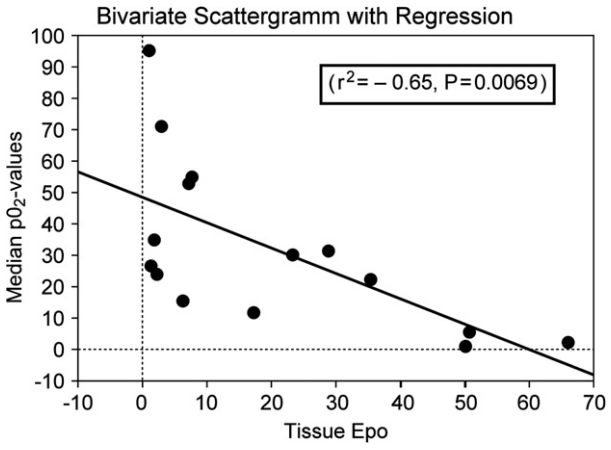

Fig. 2 – Correlations between tissue Epo and the median pO_2: the lower the median oxygen pressure, the higher the tissue Epo expression.

relationship of tumour hypoxia and the expression of EpoR and its ligand in a semiquantitative way. In the present study, we hypothesised that hypoxic tumours have high tissue Epo expression and that this relationship can be directly shown by correlating invasive polarographic oxygen measurements with quantitative analyses of tumour tissue Epo (see Figs. 1 and 2).

2. Materials and methods

2.1. Patients

Fifteen canine patients with tumours of various histologies, enrolled in a tumour hypoxia study approved by the Swiss Veterinary authorities, were included in this study. The primary tumours were localised in the oral cavity in 11 patients, on the extremities and skull in two patients each. Two or more quick frozen tumour tissue specimens were available from each patient. The site, histological diagnosis and tumour volumes are listed in Table 1.

2.2. Measurement of tumour hypoxia

Tumour oxygen partial pressure measurements were performed as previously described in dogs,[10] using an pO_2-Histograph (Helzel Medical Systems, Kaltenkirchen, Germany). The needle electrode was placed within the tumour tissue under ultrasound guidance (ATL 5000, Philips Medical Systems, Zurich, Switzerland). At least three different electrode tracks and a minimum of 50 recorded values were acquired for reliable statistical analysis.[11] Oxygenation status of individual tumours was described using the median pO_2 and the hypoxic fractions (% of pO_2 values ⩽10 mmHg, ⩽5 mmHg and ⩽2.5 mmHg, respectively). Tumour size was measured with callipers and the volume was calculated based on the formula: $\pi/6 \times \text{height} \times \text{width} \times \text{depth}$, which approximately describes the volume of an ellipsoid. The hypoxic subvolume (HSV) was calculated by the formula: volume (cm^3) × hypoxic fraction (% of pO_2-values ⩽5 mmHg).

2.3. Sample preparation

Tumour biopsies were taken immediately after the oxygen measurements, right before the start of radiation therapy and the samples were quick frozen in liquid nitrogen. The samples were stored at –80 °C until further use. For protein extraction the specimens were transferred directly from –80 °C in 1 ml of ice cold homogenisation buffer (0.27 M sucrose, 2 mM EDTA, pH 8.0, 1% NP-40, 1 mM PMSF, 1 mM aprotinin, 1 mM leupeptin, 1 mM pepstatin, and 1 mM NaVa) in a 5 cm^2 dounce homogeniser on ice: tissue was dounced 25 times with a pestle, and then transferred on a sucrose cushing. Tissue lysate was centrifuged for 10 min at 4 °C at 3000 rpm and protein concentration was measured. For analysis of plasma Epo, 3 ml of blood was collected into sterile CTAD tubes (Beckton and Dickinson Vacutainer System, France) and placed on ice. The tubes were centrifuged within 15 min at 2500*g* for 30 min at 4 °C. The resulting plasma was separated and stored immediately at –80 °C.

2.4. RIA

Plasma and tissue erythropoietin was measured using a commercial kit (Epo-Trac™ ^{125}I RIA kit, DiaSorin, USA) applying the method previously described by Glaus et al.[12] Tissue Epo was measured in normal tissues in order to establish control values in a series of canine tissues, such as lymphnode, liver, muscle and uterus.

2.5. Western blotting

Epo receptor status of analysed tumour specimens was performed by immunoblotting technique. Therefore, protein samples (100 μg/well) were resolved by denaturing electrophoresis on 10% SDS–polyacrylamide gels and transferred to a nitrocellulose membrane (Whatman GmbH, Dassel, Germany). The membrane was blocked for 2 h in 4% non-fat dry milk in PBS, 0.5% Tween 20, rinsed and subsequently

Table 1 – Descriptive tumour characteristics

Patient	Histology	Location	Volume (cm^3)	Hypoxic subvolume (cm^3)	Median pO_2 (mmHg)	Plasma Epo (mU/ml)	Tissue Epo (mU/ml)	Epo receptor
1	Fibrosarcoma	Oral cavity	50.3	24.7	5	13.6	50.5	+
2	Squamous cell carcinoma	Oral cavity	13.1	0	31	19.4	28.7	+
3	Squamous cell carcinoma	Oral cavity	9.2	0.5	22	15.0	35.4	+
4	Haemangiopericytoma	Limb	100.5	23.3	30	27.3	23.2	+
5	Fibrosarcoma	Skull	149.7	126.7	2	13.8	65.8	+
6	Malignant melanoma	Oral cavity	26.4	20.1	1	17.3	50.0	–
7	Malignant melanoma	Oral cavity	15.7	0	55	26.7	7.6	+
8	Squamous cell carcinoma	Oral cavity	0.9	0	32	18.5	1.7	+
9	Squamous cell carcinoma	Oral cavity	45.2	0	15	15.4	6.2	+
10	Fibrosarcoma	Oral cavity	13.2	0.13	11	19.8	17.0	+
11	Haemangiopericytoma	Limb	206.5	0	52	14.3	7.0	+
12	Histiocytic sarcoma	Skull	67.8	0	71	18.3	2.8	+
13	Malignant melanoma	Oral cavity	7.6	0	95	21.7	0.8	+
14	Malignant melanoma	Oral cavity	16.2	0	26	20.3	1.2	+
15	Malignant melanoma	Oral cavity	29.9	0	23	15.3	2.0	+
Normal tissue values (median 1.8)		Lymphnode					2.6	
		Liver					1.2	
		Muscle					0.7	
		Uterus					2.7	

incubated with the EpoR antibody (H-194, Santa Cruz) diluted in PBS and 1.0% Tween 20 was diluted 1:1000 overnight at 4 °C. The membrane was washed with PBS, 1.0% Tween 20 and incubated with the secondary antibody (horseradish peroxidase-conjugated goat anti-rabbit antibody, Amersham Pharmacia Biotech) diluted 1:5000 in 0.5% Tween 20 in PBS. After washing the membrane three times, the protein was detected using enhanced chemiluminescence (ECL, Amersham Pharmacia Biotech).

2.6. Statistical analyses

Description of patient data are given by mean (±SD) unless otherwise specified. The dependence of different tumour and patient characteristics on plasma and tissue Epo levels was evaluated by correlation (Wilcoxon rank test, Fisher's exact probability test). Univariate proportional hazards and multiple Cox-regression analysis were used for further testing of influences of any of the descriptors on Epo status. Distribution in HSV and tumour volumes were skewed, thus logarithmically transformed values were used rather than raw measurements. In all calculations p-values of <0.05 were considered significant. For statistical analysis StatView 5.0.1 was used.

3. Results

3.1. Plasma Epo and haematological parameters

Mean plasma erythropoietin levels were within normal limits with a mean of 18.8 mU/ml (range 13.6–27.2 mU/ml, normal: 18 mU/ml (range 0–36 mU/ml)). The haematological parameters were within normal limits with mean haematocrit of 43.5% (range: 35.5–54.0%, normal: 37–55%), haemoglobin of 15.2 g/dl (range: 12.3–10.0 g/dl, normal: 12–18 g/dl). No difference could be found for any of the parameters with respect to the histological groups.

3.2. Tumour hypoxia

The oxygen measurements were comparable to previous findings.[13] More than 20% of the values were below 10 mmHg, 16% of all readings were below 5, 13% of all readings below 2.5 mmHg, indicating severe hypoxia. The mean of all median pO_2 values was 27 mmHg (range: 0–95 mm Hg).

3.3. Tissue Epo and EpoR expression

The control values of normal canine tissue Epo expression were established and are presented in Table 1.

All of the examined tumour tissues were positive for tissue Epo ($n = 15$), and 93% of these also expressed erythropoietin receptor. Mean tissue Epo levels were 20.0 mU/ml (range: 0.8–65.8 mU/ml, normal: 0.7–2.7 mU/ml, Table 1).

3.4. Relationship between tumour hypoxia and Epo expression

Significant positive correlations were found between tissue Epo and the percentages of pO_2 values $\leqslant$10 mmHg ($r^2 = 0.93$, (95% confidence interval) 95% CI (0.79, 0.98) $p = <0.0001$), as well as the hypoxic subvolume ($r^2 = 0.86$, 95% CI (0.61, 0.95), $p = <0.0001$). Tumours with median pO_2 values $\leqslant$10 mmHg had significantly higher tissue erythropoietin levels ($r^2 = -0.65$, 95% CI (–0.87, –0.21) $p = 0.007$) than tumours with median pO_2 values >10 mmHg.

No correlations between the percentages of pO_2 values $\leqslant$10 mmHg, the median pO_2, or the level of tissue Epo and plasma erythropoietin concentrations were found.

Multivariate analysis revealed that neither of the haematological parameters nor tumour volumes influenced the amount of expression of tissue or plasma Epo levels ($p > 0.27$), while the influence of the median pO_2 and the pO_2 values $\leqslant$10 mmHg again was found to be important ($p < 0.03$).

4. Discussion

Our findings have strong implications for theories regarding Epo biology in spontaneous tumours. The level of tissue Epo but not plasma Epo expression strongly correlates with the level of tumour hypoxia, indicating a paracrine role of tissue Epo at the cellular level in the tumour.[14] The fact that stronger correlation concerning the percentages of low pO_2 values and tissue Epo, rather than the median pO_2 value, was found indicates a preferential induction of tissue Epo at very low oxygen levels. This finding is further supported by the preferential distribution of Epo staining in perinecrotic (highly hypoxic) regions.[15] Furthermore, Arcasoy and colleagues[3] describe a significant positive correlation between regional tissue Epo expression and the hypoxia-marker pimonidazole, which strongly supports the concomitance of high Epo expression in tissue regions with low pO_2. While polarographically measurable low oxygen tension in tumours is known to modulate the sensitivity of cancer cells to various treatment modalities,[5,7,16] the effect of hypoxia-induced endogenous Epo on treatment outcome has not yet been described.

Intermittent hypoxia is an effective stimulus for Epo synthesis and at high altitudes physiological plasma Epo rises rapidly, peaking at 20–48 h, thereafter declining and reaching normal level values again.[12,17]

The fact that in this study plasma erythropoietin levels did not correlate with tumour hypoxia may indicate that in a chronic condition as exerted by a neoplastic disease, either the normal values of plasma Epo have already been reestablished, and/or most of the tissue erythropoietin produced by the tumour directly binds to its local receptors and the small amounts released into the blood stream do not influence plasma erythropoietin levels. This finding, together with the coexpression of Epo and EpoR in tumour cells, is indicative of an autocrine–paracrine activation loop, presenting a potential therapeutic target in tumours where the Epo–EpoR signalling may be involved in tumour progression and angiogenesis.[4]

Similar to the findings of other studies,[3,9,15] 93% of all evaluated tumour tissues in this study were positive for EpoR. In vitro, hypoxia induces nuclear accumulation of the hypoxia inducible factor (HIF-1) protein and also upregulates EpoR-protein expression,[1,8,15] and Molhyedin et al. found in their study in HNSCC cell lines a hypoxia-inducible upregulation of EpoR rather than tissue Epo.[15] However, in this and other studies the expression of Epo-R is also found in normoxic tumours, indicating the induction through another oncogenic mechanism.[8]

Epo must act through binding of EpoR which will in turn stimulate downstream signalling in the cell through the JAK/STAT pathway.[9] However, the presence of EpoR does not guarantee its functional capacity[9] and the Western-blot analysis does not discriminate between EpoR expression localised to the cell surface and expression localised to the cytoplasmic region of the cell. Although cellular proliferation and anti-apoptotic protection at supraphamacologic concentrations of Epo has been shown in irradiated tumour cells *in vitro*,[15,18] the cellular signalling mechanism must yet be proven. The presence of a functional EpoR–Epo-system, however, may contribute to the selection of cells with diminished apoptotic potential and relative resistance to various cancer treatments,[8,18,19] and may be further enhanced by exogenous Epo administration. These *in vitro* findings and the high percentage of Epo in various canine tumour tissues offer support for the prior raised hypothesis of a negative effect of administered rHuEpo to anaemic tumour patients.[20]

In conclusion, we have demonstrated that there is a strong direct correlation between the prognostic significant polarographically measured amount of hypoxia[21] and the expression of Epo in canine malignant tissue. Since increased Epo signalling may be one of the mechanisms by which hypoxia promotes tumour aggression, the presence of Epo and EpoR needs to be quantitatively and functionally tested as novel prognostic biological parameters in neoplastic tissues.

Conflict of interest statement

The authors have no relationship, financial or otherwise, with any manufacturers or distributors of products evaluated in the paper.

REFERENCES

1. Acs G, Acs P, Beckwith SM, et al. Erythropoietin and erythropoietin receptor expression in human cancer. *Cancer Res* 2001;**61**:3561–5.
2. Arcasoy MO, Amin K, Karayal AF, et al. Functional significance of erythropoietin receptor expression in breast cancer. *Lab Invest* 2002;**82**:911–8.
3. Arcasoy MO, Amin K, Chou SC, Haroon ZA, Varia M, Raleigh JA. Erythropoietin and erythropoietin receptor expression in head and neck cancer: relationship to tumor hypoxia. *Clin Cancer Res* 2005;**11**:20–7.
4. Hardee ME, Arcasoy MO, Blackwell KL, Kirkpatrick JP, Dewhirst MW. Erythropoietin biology in cancer. *Clin Cancer Res* 2006;**12**:332–9.
5. Höckel M, Knoop C, Schlenger K, et al. Intratumoral pO_2 predicts survival in advanced cancer of the uterine cervix. *Radiother Oncol* 1993;**26**:45–50.
6. Nordsmark M, Overgaard J. Tumor hypoxia is independent of hemoglobin and prognostic for loco-regional tumor control after primary radiotherapy in advanced head and neck cancer. *Acta Oncol* 2004;**43**:396–403.
7. Höckel M, Schlenger K, Aral B, Mitze M, Schäffer U, Vaupel P. Association between tumor hypoxia and malignant progression in advanced cancer of the uterine cervix. *Cancer Res* 1996;**56**:4509–15.
8. Acs G, Zhang PJ, McGrath CM, et al. Hypoxia-inducible erythropoietin signaling in squamous dysplasia and squamous cell carcinoma of the uterine cervix and its potential role in cervical carcinogenesis and tumor progression. *Am J Pathol* 2003;**162**:1789–806.
9. Hoogsteen IJ, Peeters WJ, Marres HA, et al. Erythropoietin receptor is not a surrogate marker for tumor hypoxia and does not correlate with survival in head and neck squamous cell carcinomas. *Radiother Oncol* 2005;**76**:213–8.
10. Achermann R, Ohlerth S, Fidel J, et al. Ultrasound guided, pre-radiation oxygen measurements using polarographic oxygen needle electrodes in spontaneous canine soft tissue sarcomas. *In Vivo* 2002;**16**:431–7.
11. Höckel M, Schlenger K, Knoop C, Vaupel P. Oxygenation of carcinomas of the uterine cervix: evaluation by computerized O2 tension measurements. *Cancer Res* 1991;**51**:6098–102.

12. Glaus TM, Grenacher B, Koch D, Reiner B, Gassmann M. High altitude training of dogs results in elevated erythropoietin and endothelin-1 serum levels. *Comput Biochem Physiol A Mol Integr Physiol* 2004;**138**:355–61.
13. Achermann RE, Ohlerth SM, Rohrer Bley C, et al. Oxygenation of spontaneous canine tumors during fractionated radiation therapy. *Strahlenther Onkol* 2004;**180**: 297–305.
14. Spivak JL. The anaemia of cancer: death by a thousand cuts. *Nat Rev Cancer* 2005;**5**:543–55.
15. Mohyeldin A, Lu H, Dalgard C, et al. Erythropoietin signaling promotes invasiveness of human head and neck squamous cell carcinoma. *Neoplasia* 2005;**7**:537–43.
16. Nordsmark M, Overgaard M, Overgaard J. Pretreatment oxygenation predicts radiation response in advanced squamous cell carcinoma of the head and neck. *Radiother Oncol* 1996;**41**:31–9.
17. Eckardt KU, Kurtz A. Regulation of erythropoietin production. *Eur J Clin Invest* 2005;**35**(Suppl. 3):13–9.
18. Farrell F, Lee A. The erythropoietin receptor and its expression in tumor cells and other tissues. *Oncologist* 2004;**9**(Suppl. 5):18–30.
19. Dagnon K, Pacary E, Commo F, et al. Expression of erythropoietin and erythropoietin receptor in non-small cell lung carcinomas. *Clin Cancer Res* 2005;**11**:993–9.
20. Bohlius J, Langensiepen S, Schwarzer G, et al. Recombinant human erythropoietin and overall survival in cancer patients: results of a comprehensive meta-analysis. *J Natl Cancer Inst* 2005;**97**:489–98.
21. Rohrer Bley C, Ohlerth SM, Roos M, Wergin MC, Achermann B, Kaser-Hotz B. Influence of pre-treatment polarographically measured oxygenation levels in spontaneous canine tumors treated with radiation therapy. *Strahlenther Onkol* 2006;**182**:518–24.