The Veterinary Journal 191 (2012) 334–340

Contents lists available at ScienceDirect

The Veterinary Journal

journal homepage: www.elsevier.com/locate/tvjl

Expression of insulin-like growth factor-1 by canine insulinomas and their metastases

Floryne O. Buishand [a], Mariëlle G.M. van Erp [a], Harmke A. Groenveld [a], Jan A. Mol [a], Marja Kik [b], Joris H. Robben [a], Hans S. Kooistra [a], Jolle Kirpensteijn [a,*]

[a] Department of Clinical Sciences of Companion Animals, Utrecht University, PO Box 80154, 3508 TD Utrecht, The Netherlands
[b] Department of Pathobiology, Division Pathology, Faculty of Veterinary Medicine, Utrecht University, Yalelaan 1, 3584 CL Utrecht, The Netherlands

ARTICLE INFO

Article history:
Accepted 16 March 2011

Keywords:
Canine
Insulinoma
β-cell tumour
Growth hormone
Insulin-like growth factor

ABSTRACT

The long-term prognosis after surgical resection of canine insulinoma is poor. Signs of hypoglycaemia often recur soon after surgery because tumour tissue has only been resected partially and/or functional (micro-)metastases were present. Using quantitative real-time PCR, the expression of 16 target genes was compared between primary canine insulinomas and their corresponding metastases. There was significantly higher expression of genes encoding for growth hormone (GH) and insulin-like growth factor-1 (IGF-1) in metastases compared to their primary tumours. Immunohistochemical examination of proteins of the GH–IGF-1 axis revealed expression of GH, IGF-1 and GH receptor (GHR) in both primary insulinomas and metastases. Immunohistochemical staining for IGF-1 was significantly higher in metastases compared to primary tumours.

Introduction

Insulinomas (INS) are insulin-secreting pancreatic tumours, often associated with hypoglycaemia. Although canine INS often lack histological criteria of malignancy, >95% are malignant and they tend to metastasis to the regional lymph nodes and liver (Leifer et al., 1986; Caywood et al., 1988; Buishand et al., 2010).

Based on survival analysis, surgical resection of the primary INS and metastases, followed by additional chemotherapy, is considered to be the best treatment option compared to medical therapy alone. Reported median survival times after surgery are 381 days (Tobin et al., 1999), 435 days (Leifer et al., 1986) and 785 days (Polton et al., 2007). The longer survival time in the latter study may reflect improvements in surgical techniques. In the study of Polton et al. (2007), dogs treated with medical therapy alone had a median survival time of 196 days. Although dogs treated by surgery survive longer than medically-treated dogs, the prognosis after surgery is still guarded. Some INS are impossible to resect without the risk of major complications and most dogs with INS have (micro-)metastases that are not visible or palpable during surgery. Outgrowth of these (micro-)metastases after therapy almost always causes recurrence of hypoglycaemia (Caywood et al., 1988; Tobin et al., 1999).

In addition to surgery, or as an alternative, medical management with diazoxide, glucocorticoids, the β-cell cytotoxic agent streptozocin or the somatostatin analogue octreotide can be used to treat dogs with INS (Steiner and Bruyette, 1996; Moore et al., 2002; Robben et al., 2006). Diazoxide and glucocorticoids are symptomatic treatments intended to increase plasma glucose concentration and render temporary relief from clinical signs. Treatment with streptozocin, which selectively destroys pancreatic β-cells, is associated with acute renal failure and has an unpredictable effect on tumour growth. The effectiveness of octreotide therapy in dogs with INS is also unpredictable, possibly due to the variable expression of somatostatin receptors on neoplastic β-cells. Moreover, octreotide has a relatively short (3–4 h) suppressive effect on plasma insulin concentration in dogs and some dogs become refractory to octreotide treatment (Lothrop, 1989). Because of the disadvantages of current medical therapies, new therapeutic strategies are warranted to improve the prognosis of dogs with INS.

A novel approach in human oncology is the use of therapeutics that specifically target gene products that promote tumour growth and metastasis. For example, the growth of mammary tumours that express receptors for the growth-promoting epidermal growth factor (EGF) can be inhibited by blocking the EGF receptor (O'Donovan and Crown, 2007). Each tumour type expresses a unique combination of genes that favours tumour growth and metastasis. Therefore, in order to develop new medical therapies for INS, more knowledge is required about the genes involved in INS proliferation and spread.

In this study, the expression of 16 genes associated with β-cell proliferation, β-cell regeneration and/or β-cell differentiation was compared between primary canine INS and their metastases. The

* Corresponding author. Tel.: +31 302531698.
E-mail address: J.Kirpensteijn@uu.nl (J. Kirpensteijn).

1090-0233/$ - see front matter © 2011 Elsevier Ltd. All rights reserved.
doi:10.1016/j.tvjl.2011.03.014

16 target genes were chosen based on a review of relevant literature and on the availability of quantitative real time-PCR (qPCR) primers for these specific genes. Since pregnancy is the only physiological state where β-cell mass can increase (Bonner-Weir, 2000; Bouwens and Rooman, 2005), it was hypothesised that, similar to the situation in the mammary gland during pregnancy, the progesterone-growth hormone (GH) axis may be involved in the proliferation of canine INS. Therefore, expression of the progesterone receptor (PR), GH and its receptor (GHR), insulin-like growth factors (IGF) 1 and 2 and the IGF type 1 receptor was investigated.

Epidermal growth factor receptors ErbB1, ErbB2, ErbB3 and ErbB4 were also selected; epidermal growth factor (EGF) is a growth factor for many epithelial tissues, including the pancreas, and, together with gastrin, can stimulate proliferation and regeneration of β-cells in vivo (Bouwens and Rooman, 2005). EGF is known to stimulate phosphorylation of the PR, thereby stimulating tumour growth in breast cancer (Daniel et al., 2007).

Expression of leukaemia inhibitory factor (LIF), a member of the interleukin (IL)-6 cytokine family, and its receptor (LIFR), were also included because these factors are expressed in the normal rat pancreas and over-expressed in the regenerating rat pancreas (De Breuck et al., 2006). The transcription factors PDX-1, NeuroD1 and FoxM1 were also selected, since they are β-cell differentiation markers and play an essential role in differentiation of pancreatic precursor cells towards an endocrine cell fate (Gasa et al., 2004; Bernardo et al., 2008). FoxM1 is an important transcription factor for β-cell proliferation; loss of this factor in mice results in a postnatal β-cell replication block and causes diabetes mellitus in a few weeks (Zhang et al., 2006). Insulin (INS) was included to quantify the relative insulin production in tumour cells.

Materials and methods

Samples

Ten primary INS were collected from dogs diagnosed with INS at the Department of Clinical Sciences of Companion Animals of the Faculty of Veterinary Medicine, Utrecht University (Table 1). In addition to the primary tumours, one metastatic tumour was sampled from each of seven dogs and two metastatic tumours were sampled from each of three dogs, resulting in a total of 13 metastases (Table 2). Tumour samples for RNA extraction were carefully resected from the central portion of the primary INS and metastases to avoid contamination of samples by healthy tissue adjacent to the neoplastic tissue. For all primary and metastatic tumour samples, the presence of neoplastic β-cells was confirmed by histological examination. The tumour specimens (5–15 mm in diameter) were frozen in liquid nitrogen immediately after surgical removal and stored at −70 °C until RNA was extracted.

RNA extraction and cDNA synthesis

Total RNA was extracted using the RNeasy Mini Kit (Qiagen). An on-column DNase treatment was included to prevent contamination of the samples with genomic DNA. RNA concentrations were measured by spectrophotometry (NanoDrop ND-1000, Isogen Life Sciences) and 2 μg total RNA was reverse transcribed in a total reaction volume of 40 μL to create enough volume of copy DNA (iScript cDNA Synthesis Kit, Bio-Rad). Reactions without the reverse transcriptase enzyme (RT minus reactions) were performed for all samples.

Quantitative real-time PCR

cDNA samples were diluted 1:4 with milliQ water, divided into 2 μL aliquots and stored at −20 °C. For each target gene, a master mix was prepared using SYBR Green Supermix (Bio-Rad). qPCR was performed using a MyiQ Single Color Real-Time PCR Detection System (Bio-Rad), with RPL8 as an endogenous reference gene. Each reaction was performed in duplicate, and efficiency was assessed by a dilution series of pooled cDNA samples in each run. The RT minus reactions tested for the reference gene showed no significant contamination with genomic DNA. For primers with an annealing temperature (T_a) < 58 °C, three-step reactions (denaturation, annealing and elongation) were performed as described by Brinkhof et al. (2006). For primers with an annealing temperature ⩾58 °C, two-step reactions (denaturation and annealing) were performed (Brinkhof et al., 2006). Primers were designed using PrimerSelect version 5.05 (DNAStar) and optimal annealing temperatures were determined by qPCR using a gradient annealing temperature (Table 3). The amplified products were verified by sequencing.

Immunohistochemistry

Since mRNA expression does not automatically correlate with protein levels, immunohistochemistry was performed to demonstrate protein expression. For 8/10 dogs included in this study, paraffin-embedded blocks of both primary INS and metastases were available for immunohistochemistry. Immunohistochemistry was performed on proteins of the GH/IGF-1 axis, since qPCR showed that expression of genes of this axis was significantly different between primary INS and metastases.

After deparaffinisation and antigen retrieval, slides were rinsed in Tris-buffered saline containing Tween-20 (TBST), then blocked for 30 min with 10% normal goat serum. Sections were incubated overnight at 4 °C with primary antibodies: polyclonal rabbit anti-porcine GH antibody (generous gift from Dr. M.M. Bevers, Department of Farm Animal Health, Utrecht University) at a dilution of 1:5000, polyclonal rabbit anti-human IGF-1 antibody (kindly donated by Dr. A.F. Parlow, NHPP) at a dilution of 1:1000 and a monoclonal rat/rabbit GH receptor antibody (Mab 263, Abcam) at a dilution of 1:75. After incubation, slides were washed with TBST and sections were treated with 0.35% H_2O_2 in Tris-buffered saline (TBS) for 15 min to block endogenous peroxidase activity. Slides were washed again with TBST and then incubated for 30 min with Envision Anti-Rabbit (Dako). After another wash with TBST, the antigen–antibody reaction was visualised following addition of 3,3′-diaminobenzidine. Slides were counterstained with haematoxylin. Sections were dehydrated through ascending ethanol series and xylene and mounted with cover slips over Vectamount-permanent mounting medium.

To score the immunoreactivity of GH, IGF-1 and GHR, staining intensity was graded as: 0, negative; 1+, weak; 2+, moderate and 3+, strong (Fig. 1). When heterogeneous staining was present, scoring was based on the mean intensity. The proportion of positive INS cells was graded as: 0, negative; 1+, <10%; 2+, <24%; 3+, 25–49%; 4+, 50–74%; 5+, 75–100%.

Statistical analysis

qPCR data were analysed with iQ5 software (Bio-Rad); gene expression was normalised against the expression of RPL8. REST-384 version 1 (Pfaffl et al., 2002) was used to compare the mean Ct values of the groups assessed by the pair-wise fixed reallocation test, with 2000 randomisations to correct for multiple testing. P values < 0.05 were considered to be significant. For the REST calculations, the mean

Table 1
Case details of hypoglycaemic dogs with insulinomas.

Dog	Breed	Sex	Age (years)	Glucose (mmol/L)	Diameter of tumour (mm)	TNM stage
A	Bearded Collie	Fx	9	2.7	15	III
B	Crossbreed	Fx	12	1.7	35	IV
C	Labrador Retriever	Mx	10	1.9	40	III
D	Labrador Retriever	Mx	6	2.0	3[a]	III
E	Belgian Shepherd Dog	Mx	10	2.2	15	III
F	Crossbreed	Fx	9	3.2	8	III
G	Collie	Mx	10	2.0	50[b]	IV
H	Golden Retriever	Mx	13	2.0	25	IV
I	Anatolian Shepherd Dog	Mx	6	2.0	90[a]	IV
J	Boxer	Fx	9	2.6	25	III

Mx, castrated male; Fx, castrated female; tumour-node-metastasis (TNM) staging performed according to Buishand et al. (2010).
[a] Multiple insulinoma nodules present, diffusely spread through the left pancreatic lobe.
[b] Two primary insulinomas present.