Expression of angiogenesis-related genes in canine cortisol-secreting adrenocortical tumors

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ABSTRACT

The aim of this study was to evaluate the expression of angiogenesis-related genes in canine cortisol-secreting adrenocortical tumors (ATs). Quantitative RT-PCR analysis revealed mRNA encoding for vascular endothelial growth factor, vascular endothelial growth factor receptors 1 and 2, angiopoietin 1 and 2 (*ANGPT1* and *ANGPT2*), the splice variant $ANGPT2_{443}$, the ANGPT-receptor *Tie2*, and basic fibroblast growth factor in 38 canine cortisol-secreting ATs (26 carcinomas and 12 adenomas) and 15 normal adrenals. The relative expression of both *ANGPT2* and $ANGPT2_{443}$ was higher in adenomas ($P = 0.020$ for ANGPT2 and $P = 0.002$ for ANGPT2$_{443}$) and carcinomas ($P = 0.003$ for ANGPT2 and $P < 0.001$ for ANGPT2$_{443}$) compared with normal adrenals, and this enhanced expression was also detected with Western blot analysis. Immunohistochemistry indicated expression of ANGPT2 protein in AT cells and in vascular endothelial cells of carcinomas, whereas Tie2 was mainly present in the tumor vascular endothelial cells. The *ANGPT2*-to-*ANGTPT1* ratio, a marker for a proangiogenic state, was higher in both adenomas ($P = 0.020$) and carcinomas ($P = 0.043$). With the use of the human H295R cortisol-producing adrenocortical carcinoma cell line, we were able to demonstrate that the *ANGPT2* expression was stimulated by cyclic adenosine monophosphate and progesterone but not by cortisol. In conclusion, canine cortisol-secreting ATs have enhanced ANGPT2 expression with a concomitant shift toward a proangiogenic state. On the basis of this information, treatment modalities may be developed that interfere with ANGPT2 expression, including inhibition of the cyclic adenosine monophosphate/protein kinase A pathway, or of the effect of ANGPT2, by using specific ANGPT2 inhibitors.

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1. Introduction

Adrenocorticotropin (ACTH)-independent hypercortisolism in dogs as a result of autonomous glucocorticoid production by an adrenocortical tumor (AT) accounts for approximately 15% of cases of spontaneous canine hypercortisolism [1]. The treatment of choice is adrenalectomy, because the successful complete removal of the affected adrenal gland will eliminate the clinical signs related to glucocorticoid excess without the need for lifelong medication. However, invasive growth in the surrounding tissues or metastasis or both may preclude complete removal. Factors involved in tumor growth and metastasis of canine cortisol-secreting ATs are largely unknown.

In general, angiogenesis, the process of new blood vessel formation from existing vasculature, is an important factor in tumor development and metastasis. By means of intratumoral angiogenic feedback loops, tumors may activate angiogenesis and provide themselves with the nutrients and oxygen necessary to grow beyond a certain size [2]. In human cancer research, the use of antiangiogenic drugs is one of the most rapidly emerging therapeutic strategies [3]. The aim of the present study was to evaluate the expression of angiogenesis-related genes in canine

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cortisol-secreting ATs. Knowledge on the role of angiogenesis in AT development may help in the development of new treatment modalities.

In the regulation of tumor angiogenesis and the development of antiangiogenic drugs, basic fibroblast growth factor (bFGF), the vascular endothelial growth factor (VEGF) family, and the angiopoietin (ANGPT) family play a pivotal role [2,4]. Although inhibition of bFGF is still in the early stages of development, its role as a potent mitogen for fetal and adult adrenocortical cells [5,6] and its increased expression in human adrenal medullary tumors [7] make bFGF a target of specific interest in adrenal gland pathology.

Drugs that target VEGF have shown potential in various clinical and preclinical trials, inhibiting angiogenesis and tumor growth [2,8]. Furthermore, high VEGF expression has been associated with increased microvascular density in human adrenocortical carcinoma [9].

Angiopoietin signaling has likewise been implicated as a significant factor in the pathogenesis of human ATs [10]. Especially, the ratio between ANGPT1 and ANGPT2 is considered an important indicator of activation of the angiogenic switch in tumors [11]. Selective ANGPT2 inhibition, either combined with VEGF inhibition or by itself, has shown promise in slowing tumor angiogenesis and tumor growth in different tumor types [12,13]. The expression of ANGPT2 in the human adrenal gland is thought to be regulated by ACTH–cyclic adenosine monophosphate (cAMP)–steroidogenic factor 1 signaling [14,15]. Recently, we demonstrated that a large proportion of canine cortisol-secreting ATs harbors an activating mutation in guanine nucleotide binding protein, alpha stimulating, the gene responsible for cAMP production on ACTH-stimulation [16]. Data about the relation between cAMP signaling and angiogenesis in canine ATs are lacking.

The aim of the study was to evaluate the expression of bFGF, VEGF, VEGF receptors 1 and 2, ANGPT1 and ANGPT2, the splice variant ANGPT2$_{443}$, and the ANGPT-receptor Tie2 in canine cortisol-secreting carcinomas and adenomas, compared with that in normal adrenals. In addition, we investigated whether the expression of genes of interest was influenced by cAMP or the adrenocortical hormones cortisol and progesterone in vitro.

2. Materials and methods

2.1. Patient material

In this study 38 canine cortisol-producing ATs and 15 normal adrenal glands were used. Adrenal glands from healthy dogs were available as archived tissue for comparison with AT tissue obtained from patients. After surgical removal of an AT in the patients or resection of a normal adrenal gland in the healthy dogs, the tissue was stored on ice and inspected, and material was saved for quantitative RT-PCR (qPCR) analysis and histopathology. The fragments for RNA isolation were cut and snap-frozen in liquid nitrogen within 10 min. They were kept at $-80°C$ until further use. The remaining part of the AT tissue was immersed in formalin for fixation and embedded in paraffin after 24 to 48 h. The tumor group consisted of all histologically confirmed ATs derived from patients referred to the Department of Clinical Sciences of Companion Animals of the Faculty of Veterinary Medicine in Utrecht between 2001 and 2009 with clinical signs of hypercortisolism. The diagnosis of ACTH-independent Cushing's syndrome because of an AT was based on elevated urinary corticoid-to-creatinine ratios, which were not suppressible with high doses of dexamethasone; suppressed or even undetectable plasma ACTH concentrations [1]; and demonstration of an AT by ultrasonography or computed tomography [17]. All dogs underwent unilateral adrenalectomy. The dogs' ages at the time of surgery ranged from 6 to 14 y (mean, 9 y). Twelve dogs were mongrels, and the other dogs were of 10 different breeds. Eighteen dogs were male (8 castrated) and 20 female (15 neutered). The ages of the control dogs ranged from 2 to 5 y. Five of the control dogs were male and 10 were female; all control dogs were intact. Permission to use the AT tissue for this study was obtained from all patient owners, and the study was approved by the Ethical Committee of Utrecht University.

2.2. Histopathology

Histopathologic evaluation was performed on formalin-fixed and paraffin-embedded tissue slides of all samples and used to confirm the diagnosis and to classify the tumors. All histologic evaluations were performed by a single pathologist. Classification was performed on the basis of the criteria described by Labelle et al [18]. Classification as a carcinoma was based on histologic evidence of vascular invasion, peripheral fibrosis, capsular invasion, trabecular growth, hemorrhage, necrosis, and single-cell necrosis. Typical histologic characteristics of adenomas were hematopoiesis, fibrin thrombi, and cytoplasmic vacuolization. On the basis of these criteria, the tumor group consisted of 12 adenomas and 26 carcinomas.

2.3. Total RNA extraction and reverse transcription

Total RNA for qPCR analysis was isolated from tissue and cell culture samples with the use of the RNeasy mini kit (Quiagen, Hilden, Germany), according to manufacturer's protocols. An optional DNAse step was performed to avoid DNA contamination. RNA concentrations were measured on the NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE, USA). Synthesis of cDNA was performed with the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA), according to manufacturer's protocols. For all samples, 1 cDNA reaction was performed without reverse transcriptase, to check for contamination with genomic DNA.

2.4. Quantitative RT-PCR

Quantitative RT-PCR primers for all target genes were designed with DNA-star primer select version 8.1, Oligoexplorer version 1.1.0, or Perl-primer version 1.1.14 according to the parameters in the Bio-Rad iCycler manual and were ordered from Eurogentec (Maastricht, The Netherlands). Primers for distinguishing both *ANGPT2* variants were designed to anneal to areas of the transcript unique to each isoform. For each primer pair a qPCR temperature gradient was performed, to determine the optimal