CHAPTER 9

Expression of leukemia inhibitory factor (LIF) and LIF-receptor in the canine pituitary gland and corticotroph adenomas

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Abstract

Leukemia inhibitory factor (LIF) is an important factor for corticotroph cell lineage differentiation and for expression and secretion of adrenocorticotropic hormone (ACTH) in the adult pituitary gland. The aim of this study was to investigate the expression of LIF and LIFRα mRNA and protein in the normal canine pituitary and in corticotroph adenomas, and to perform a mutation analysis of LIFR.

The LIF and LIFR proteins were localized by immunohistochemistry in pituitary glands of control dogs and in adenomatous tissue collected through hypophysectomy in dogs with pituitary-dependent hyperadrenocorticism (Cushing's disease). Quantitative expression analyses were performed for LIF and LIFR, and cDNA from adenomateous tissue from 14 dogs with corticotroph adenomas was screened for mutations.

There was a low magnitude of LIF expression in *pars distalis adenohypophysis* (pars distalis) of control and in tumor tissue. Immunoreactivity in the *pars intermedia adenohypophysis*) (intermediate lobe, pars intermedia) was limited to a few positive cells. In contrast to this, LIFRα was highly expressed in the intermediate lobe. In the pars distalis the LIFR co-localized with ACTH. Immunoreactivity of LIFRα was preserved in corticotroph adenomas and adjacent non-tumorous cells of pars intermedia. Surprisingly, a nuclear/perinuclear immunoreactivity to LIFR was seen in non-neoplastic cells of pars distalis in the presence of a corticotroph adenoma. No mutation was found on mutation analysis of the complete LIFRα cDNA.

These data show that LIFRα is highly co-expressed with ACTH and α-MSH in canine control pituitaries and corticotroph adenomas and that nuclear immunoreactivity for LIFRα in non-tumorous cells of pars distalis may indicate presence of a corticotroph adenoma.

In the dog, pituitary-dependent hyperadrenocorticism (PDH), in humans called Cushing's disease, is a commonly encountered endocrinopathy, caused by a corticotroph adenoma.[44] These adenomas are generally characterized as benign, slow growing pituitary tumors with increased ACTH secretion and reduced sensitivity to glucocorticoid feed-back.[7,25,44] The underlying pathogenesis is still not known. In the present study the candidate genes leukemia inhibitory factor (LIF) and its receptor are being studied. As its name indicates, LIF inhibits the proliferation of leukemias, but acts as a growth factor for several neoplasms.[6,35] For example, LIF stimulates breast cancer proliferation [21] and the proliferation of multiple myelomas.[58]

Leukemia inhibitory factor (LIF) is multifunctional glycoprotein cytokine belonging to the interleukin- 6 (IL-6) family of hemato- and neuropoeietic cytokines.[6,18,22,37,41] In human pituitaries, LIF is expressed in the normal adult, fetal as well as in tumorous tissues [5] where it has important neuroimmune mediating effects on the hypothalamic-pituitary-adrenal axis. The leukemia inhibitory factor induces POMC transcription and ACTH secretion in the corticotroph cells where LIF also acts synergistically with CRH.[2,3,5,10,42,43,46,48] LIF promotes corticotroph cell differentiation and, in this case, counteracts CRH's mitogenic effects.[48] Also, during embryogenesis, LIF has a stimulatory effect on corticotroph cell differentiation,[4,56] and early pituitary directed transgenic overexpression of LIF results in corticotroph hyperplasia and Cushingoid symtoms.[56]

The LIF receptor (LIFR) belongs to the hematopoietic cytokine receptor family, which is a member of the immunoglobulin superfamily.[22,56] Among the hematopoeitins, LIF belongs to the interleukin-6 (IL-6) family, of which all family members shares a signal transducing protein in common, the gp130.[1,12,22,50,56] There are two forms of LIFR, the membrane-bound form LIFRα and a soluble form sLIFR, which has antagonistic effects.[31,57] When the LIFRα binds to its ligand, the LIF-LIFRα complex heterodimerizes with gp130 which is a signalling transducing protein shared by all members of the IL-6 family.[1,12,22,50,56] The LIFR activates several signalling pathways in different cell types, including the Janus protein tyrosine kinase (Jak)- signal transducer and activator of transcription (STAT3), the mitogen-activated protein kinase pathway, and the phosphoinositol 3-kinase pathway.[6,16] In the pituitary, the LIFR-gp130 heterodimer activates POMC gene transcription through the Jak-STAT signal pathways requiring STAT1 and STAT3.[9-11,42] This activation appears to involve both direct binding of the POMC promoter by STAT proteins and a DNA-binding independent mechanism.[10,38] Recently, it was shown that STAT reverses glucocorticoid-dependent POMC gene inhibition.[30]

LIF has been detected in both human and murine pituitaries, and LIFR transcripts has been detected in human pituitary adenomatous tissue.[26,46] However, presence of the protein has only indirectly been demonstrated through demonstration of LIF-binding sites on the surface of cultured human pituitary cells[5]. The expression of LIF in the canine pituitary has so far not been studied in the dog. With the hypothesis that LIF and its receptor may play a role in the pathogenesis of corticotroph adenomas, we present here a study on LIF and LIFR in canine pituitaries and corticotroph adenomas including immunoreactivity for LIF and LIFR, qPCR expression profiles and a mutation analysis for LIFR in canine corticotroph adenomas.

Materials and Methods

Tissue

Pituitary specimens from 24 dogs with PDH were collected at the time of transsphenoidal hypophysectomy[33,34] for the treatment of PDH. There was 1 Beagle, 1 Bearded Collie, 1 Bernese Mountain Dog, 1 Boxer, 2 Dachshunds, 1 Dutch Shepherd, 3 English Cocker Spaniels, 1 Golden Retriever, 1 Havanese, 1 Irish setter, 1 Jack Russel Terrier, 1 Labrador Retriever, 1 Maltese, 1 Minature Poodle, 1 Shih Tzu, 1 Siberian Husky, 1 Soft Coated Retriever, 1 Vizsla, 1 Welsh Springer Spaniel, and 2 Crossbred dogs. There were 14 female dogs (9 spayed), 10 males (3 castrated), median age was 9 years (range, 3-12), and median body weight was 17 kg (range, 3.7-48 kg). The diagnosis of pituitary-dependent hyperadrenocorticism was based upon the averaged urinary corticoid-to-creatinine ratio in two consecutive morning urine samples combined with a high-dose dexamethasone test, as described earlier.[45,49] Based on the pituitary height-to-brain area ratio (P/B) (nonenlarged pituitaries, $< 0.31 \times 10^{-2}$ mm^{-1})[29], 21 dogs had enlarged pituitaries. All dogs went into initial remission after surgery.

For immunoreactive studies control pituitary tissue was collected from 2 female Greyhounds (11 and 12.5 years old) and one female and one male Labrador retriever (3.5 and 3 years old). The pituitary from one of the Greyhounds was harboring a corticotroph hyperplasia. For protein-blot analysis control pituitary tissue was used from one 12-year-old, male Greyhound and one 2-year-old Labrador Retriever. For PCR experiments, control pituitary tissue was collected from 13 laboratory dogs. The dogs (6 female dogs, 6 male dogs were 2 Beagle dogs, 1 Greyhound, 7 Labrador retrievers, and 3 Crossbred dogs. The age ranged from 0.5 to 12.5 years and the body weight ranged from approximately 20 to 25 kg. The dogs were euthanized in other experiments which have been approved by the Ethical Committee of the Faculty of Veterinary Medicine, Utrecht University, The Netherlands.

Immunohistochemistry analysis

Pituitary tissue specimen from control pituitaries and pituitary adenomas were fixed in 4% buffered paraformaldehyde and embedded in paraffin. Serial 3 µm-thick sections were mounted on silane-coated slides, deparaffinized in xylene and rehydrated in graded alcohol to PBS (137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, pH 7.4). Slides were blocked for 60 min using 10% normal goat serum (DAKO, Glostrup, Denmark) (LIFR) or 10% normal horse serum (Santa Cruz, Tebu-bio, Heerhugowaard, The Netherlands) (LIF and ACTH) followed by incubation with avidin/biotin blocking kit (Vector Laboratories, Peterborough, UK). The slides were incubated over-night with primary antibodies; anti-human-LIFR rabbit polyclonal antibody (C19, Santa Cruz) (diluted 1:100) which is raised against a peptide mapping at the C-terminus of the receptor that is shown to be highly homologous with the canine LIFR sequence[24]. The anti-human-LIF goat polyclonal antibody (N18, Santa Cruz) (diluted 1:50) was raised against an epitope mapping at the N-terminus of the cytokine. The anti-$ACTH_{1-24}$ was a monoclonal antibody (diluted 1:100) (Department of Infectious Diseases and Immunology, Faculty of Veterinary Medicine, Utrecht University, Utrecht, The Netherlands). Following incubation with primary antibody, slides were reacted with biotin-labeled anti-rabbit, anti-goat and anti-mouse IgG, respectively, incubated with ready-to-use streptavidin-horseradish peroxidase (HRP) (Vector Laboratories) and detected with 3, 3'-diaminobenzidine (DAB) (Vector Laboratories) which was followed by

enhancement with DAB enhancing solution (Vector Laboratories). The slides were counterstained with methyl green (Vector Laboratories), dehydrated, mounted with Vecta-Mount mounting medium (Vector Laboratories) and photographed. The LIF and LIFR antibodies were tested for specificity with the use of a specific blocking peptide (LIFR) and protein-blot analysis. Negative controls were performed by omitting the primary antibody. Immunoscoring was performed by multiplying the score of the stained cells (1-4), staining intensity (1-4) and staining homogeneity (1-4) according to the protocol published by Kontogeorgos and co-workers 2000[24].

Immunofluorescence analysis

Co-localization studies were performed for LIF and LIFR with ACTH. Serial 3 µm-thick sections were mounted on silane-coated slides, deparaffinized in xylene and rehydrated in graded alcohol to PBS (137 mM NaCl, 2.7 mM KCl, 6.5 mM Na_2HPO_4, and 1.5 mM KH_2PO_4, pH 7.4). The slides were transferred to an Antigen Unmasking Solution (Vector Laboratories) and heated in a microwave oven (850W) for 5 min followed by 20 min of cooling down. Blocking was performed during 60 min with 10% normal chicken serum (Santa Cruz) (LIF), 10% normal goat serum (DAKO, Glostrup, Denmark) (LIFR/ACTH). The slides were incubated over-night with anti-LIF (1:50) or anti-LIFR (1:20) antibodies as described above, then washed twice in PBS. For LIF detection, the slides were incubated with the secondary antibody Alexa Fluor 488 chicken-anti-goat IgG (diluted 1:100) (Invitrogen, Breda, The Netherlands), followed by a washing step, blocking with 10% normal goat serum and incubation with anti-ACTH antibodies for 60 min., the slides were then incubated with TOPRO-3 (diluted 1:1000) (Invitrogen) for 30 min For LIFR detection, the slides were incubated with anti-ACTH antibodies for 60 min, and after washing, incubated for 60 min with secondary antibodies, Alexa Fluor 488 goat-anti-rabbit IgG (diluted 1:125) (Invitrogen) and Alexa Fluor 568 conjugated goat-anti-mouse IgG (diluted 1:150) (Invitrogen). After a washing step nuclear staining was performed with TOPRO-3 (diluted 1:1000) (Invitrogen) for 30 min, followed by washing and mounting (Fluorsave, Calbiochem, San Diego, USA). The stained slides were stored in the dark at 4°C and photographed with a Leica TCS SP Confocal Laser Scanning Microscope (Leica Microsystems B.V., Rijswijk, The Netherlands). Single stainings with primary antibody with LIF, LIFR and ACTH, respectively, were performed as controls. Negative controls were performed by omitting the primary antibody.

Protein blot analysis

Pituitary tissue was snap-frozen in liquid nitrogen and stored at –70°C. The pituitary tissue was homogenized in RIPA buffer containing 1% Igepal, 0.6 mM phenylmethylsulfonyl-fluoride, 15 µg/ml aprotinine, and 1 mM sodium-orthovanadate (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands). Protein concentrations were determined using a Lowry-based assay with DC Protein Assay Reagents (Bio-Rad, Veenendaal, The Netherlands). Approximately 20 µg of protein was loaded per well. The supernatant was denatured for 3 min at 95°C and electrophoresed on 10-12% Tris-HCL polyacrylamide gels (Bio-Rad, Veenendaal, The Netherlands). A standard of recombinant proteins were used, Precision Plus Protein Standards (Bio-Rad). The proteins were transferred onto Hybond-C Extra Nitrocellulose membranes (Amersham Biosciences, Roosendaal, The Netherlands) using a

Mini trans-Blot Cell blot-apparatus (Bio-Rad). Immunodetection was based on an ECL Western blot analysis system, performed according to the manufacturer's instructions (Amersham Biosciences). The membranes were incubated with 4% ECL blocking solution in tris buffer saline (TBS) (0.01 mM Tris-HCl, 150 mM NaCl, pH 8.0) supplemented with 0.1% Tween-20 (TBST) (Boom BV, Meppel, The Netherlands) for 1 hour under gentle shaking. The incubation of the primary antibody was performed at 4°C over-night for both antibodies in TBST with 4% BSA (Sigma-Aldrich Chemie BV, Zwijndrecht, The Netherlands). After washing, the membranes were incubated at room temperature for 1 h with their respective HRP-conjungated secondary antibody (1:20 000) (anti-rabbit-HRP for LIFR (R&D systems, Abingdon, UK) and chicken anti-goat-HRP for LIF (Santa Cruz)). The membranes were developed in ECL Advance solution (Amersham Biosciences) and exposed to Kodak BioMax Light-1 films (Sigma-Aldrich Chemie BV). The blots were digitalized using a Canonscan D660U scanner (Canon, Amsterdam, The Netherlands).

RNA isolation and cDNA synthesis

After removal, pituitary tissue was quick-frozen in liquid nitrogen and stored at –70°C. Total RNA was extracted from frozen pituitary adenomatous tissue from 14 dogs with PDH using the RNeasy Mini Kit according to the manufacturer's instructions including the optional on-column DNase digestion (Quiagen, Leusden, The Netherlands). RNA was quantified spectrophotometrically using Nanodrop ND-1000 (Isogen Life Sciences, IJsselstein, The Netherlands) and 1 µg was reverse transcribed in a total volume of 20 µl. For mutation analysis of 6 adenomatous specimens oligo-T primers and AMV Reverse Transcriptase were used according to the manufacturer's instructions (Promega, Leiden, The Netherlands). The RT reaction was performed at 42° for 60 minutes. For mutation analysis of 8 adenomatous specimens, normal tissue, and for all tissue included in the qPCR analysis, cDNA synthesis was performed using oligo (dT) primers combined with random hexamer primers and an M-MLV-derived reverse transcriptase according to the manufacturer's instruction (iScript cDNA Synthesis Kit, Bio-Rad, Veenendaal, The Netherlands). An additional DNase step with TURBO DNA-free (Ambion , Applied Biosystems, Nieuwerkerk, The Netherlands) according to manufacturer's instructions was performed on each sample included in the qPCR analysis.

Mutation analysis

PCR was performed on 1 µl cDNA in a reaction of 1 x supplied reaction buffer, 1.5 mM $MgCl_2$, 0.2 mM dNTPs, 0.2 µM of each primer, and 2.5 U Recombinant Platinum Taq polymerase (Invitrogen, Breda, The Netherlands), at an experimentally determined optimal annealing temperature for each primer pair, using MiniCycler units (MJ Research Inc, Bio-Rad, Veenendaal, The Netherlands). The products were visualized on ethidium-bromide containing 1.5% agarose gels. Previously published primers covering the whole coding sequence for canine LIFR were used for sequencing analysis[24]. Additionally, two primer pairs were developed using PrimerSelect software version 5.05 (DNASTAR Inc., Madison, WI); 5'-ACTGACTACTTTTGCACGGATGAT-3' and 5'-AACCCCTGTCATTCCA-CTTT-3' were used for additional amplification of the 5'end of LIFR cDNA. The non-intron-spanning primers 5'-TCTGATGCGGAAGCTGAGAA-3' and 5'-GAGCTCACTG-

AGATGGCAGA-3' were used for complementary amplification of the 3'end of LIFR. The amplified products were verified by sequencing.

The PCR products were diluted 1:10 in distilled water and 1-2 µl of the dilution was used in the sequence reaction using BigDye Terminator Cycle Sequencing Ready reaction (Applied Biosystems, Foster City, CA), according to the standard protocol. The tercycle reaction consisted of 25 cycles each of 30 sec at 96° C, of 15 sec at 50-53° C and of 2 min at 60°C. The Tercycle product was purified using multiscreen 96-well filtration plate (Millipore, Amsterdam, The Netherlands), and analyzed on an ABI3100 Genetic Analyzer (Applied Biosystems, Foster City, CA). Mutation analysis was performed with the Seqman software (DNASTAR Inc., Madison, WI).

Quantitative PCR

Quantitative PCR was performed using a Bio-Rad My-IQ detection system (IQ SYBR green Supermix and My-IQ (Bio-Rad, Veenendaal, The Netherlands) according to manufacturer's instructions, with a final primers-concentration of 400 nM each, and 0.5 µl cDNA template per reaction. LIF was amplified using a 3-step program and LIFR was amplified using a 2-step program. Optimal annealing temperature was determined experimentally, and the amplified products were confirmed by sequencing as described above. Efficiency for each reaction was determined by dilution of pooled cDNA samples and tested for each replicate run. No efficiency was below 90%. Analysis was performed with My-IQ software (Bio-Rad, Veenendaal, The Netherlands). The LIF primers used were non-intron-spanning 5'-GAGCCCCCTTCCTATCAC and 5'- CCAGCCGGGTCTTCTCC-3' located in exon 3. For LIFR an intron-spanning primer-pair, 5'-ACTGGAGTTGGACCTCAGAC-3' located in exon 6 and 5'-CTGAGAATCAGGTGACCAAG-3'located in exon 6 and 7.[24] The ribosomal protein S19 and hypoxanthine phosphhoribosyltransferase (HPRT) were used as reference genes.[13] Additionally, the pituitary-specific T-box Tbx19 (Tpit) was used as an marker for pro-opiomelanocortin (POMC) expressing cells (corticotrophs and melanotrophs.[23,53] Presence of high POMC expression in relation to GH expression in the adenomatous material was confirmed with quantitative PCR (data not shown).

The cycle threshold (Ct) values of LIF and LIFR were normalized to the Ct values of the reference genes, using the formula described by Muller and co-workers[36]. The expression of LIF was normalized to RPS19 and HPRT and the expression of LIFR was normalized to RPS19, HPRT and Tpit was used as reference genes. The normalized expression data were then analyzed using SPSS software (SPSS Benelux BV, Gorinchem, the Netherlands). For data that were not normally distributed, the non-parametric Mann-Whitney test was used.

Results

Spatial localization of the LIFR and LIF proteins

The immunoreactivity for LIF was diffusely cytoplasmatic. In control pituitary tissues, most cells of the pars distalis were immunopostitive for LIF protein to a varying but mainly low degree of intensity, and only partially co-localized with ACTH. (Figure 1A, D). In the pars tuberalis a perinuclear staining pattern was seen. The pars intermedia was almost completely immunonegative for LIF except for a few positive cells (Figure 1B). The distribution and degree of staining intensity varied greatly in corticotroph adenomas (score 1-32), in 4/13

adenomas LIF, was almost undetectable. Most tumors (9/13) showed heterogenous distribution of LIF with variable staining intensity and clusters of adenoma cells with stronger LIF immunoreactivity. In one tumor, LIF showed an intranuclear staining pattern. The immunoreactivity of the non-tumorous tissue, if available, showed an equal or higher staining intensity than the tumorous tissue. Thus, only a subpopulation of the ACTH positive tumor cells stained positive for LIF (Figure 1E, F).

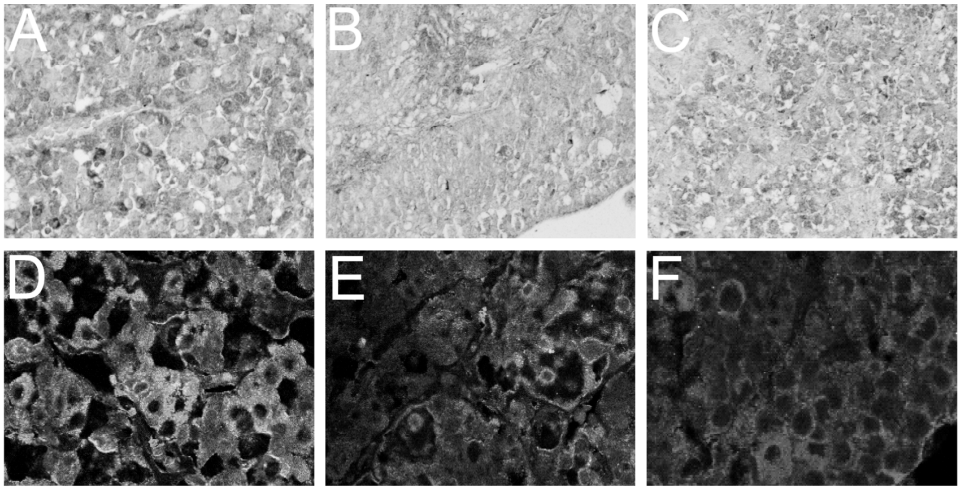

Figure 1. Immunoreactivity to leukaemia inhibitory factor (LIF) detected with DAB (brown) in the *pars distalis adenohypophysis* (A), and *pars intermedia adenohypophysis* (B) of a control dog, and a pituitary adenoma (C). Counterstaining was performed with methyl green. Immunofluorescence reaction for LIF (green) and ACTH (red) in the *pars distalis adenohypophysis* of a control dog (D), and a corticotroph adenoma infiltrating normal tissue (E) and tumerous tissue from a second corticotroph adenoma (F) (Magnification 400 x).*LIFR*

The LIFR was strongly co-expressed with the POMC-expressing cells in both the pars distalis and the pars intermedia (Figure 2, 3). In pituitaries harboring an adenoma, the expression of LIFR followed that of POMC (Figure 4). Non-tumorous cells of the pars distalis were immunonegative, but parts of the pars intermedia were still positive, similar to what was seen for POMC. Additionally, an intranuclear to perinuclear immunoreactivity for LIFR was present in non-tumorous pituitary cells of the pars distalis in all (10/10) available tissue specimes from PDH dogs (Figure 4). This was not observed in the normal control tissues or in the pituitary adenomas with corticotropic hyperplasia. One dog showed both low LIF and LIFR.

Protein blot analysis

The calculated molecular weight of the LIFR protein is 124 kDa. The mature forms of both mouse and human LIF are highly glycosylated molecules with the glycosylation moyeity varying in molecular weight from 38-67 kDa.[6] The expected size of LIFR on protein blot was

about 190 kDa.[1] Additional specific immunoreactive bands were seen at the size of 80 and 90 kDa (Figure 5).

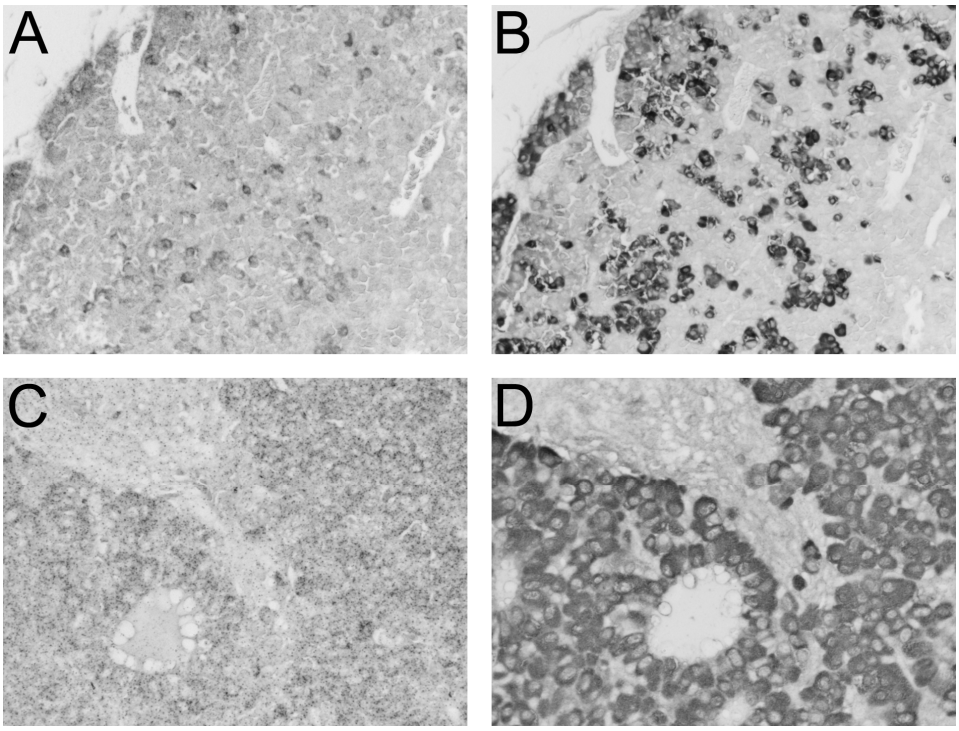

Figure 2. Immunoreactivity detected with DAB (brown) for leukemia inhibitory factor (LIFRα) (A and C) and adrenocorticotropic hormone (ACTH) (B and D) in the *pars distalis adenohypophysis* (A and B) and the *pars intermedia adenohypophysis* (B and D) of a control dog (Magnification 400 x).

RT-PCR reaction and mutation analysis

LIFR was expressed in all 14 adenomatous tissues investigated. No mutations were found on full-length cDNA mutation analysis of 14 corticotroph adenomas. However, a few heterozygote SNP's were found that did not result in different amino acids. When BLASTed to the canine genome available at the National Center for Biotechnology Information (www.ncbi.nlm.nih.gov/BLAST), the sequence of the canine LIF gene is located at chromosome 26 in close vicinity to the oncostatin M (OSM) gene which was identified by entering the human OSM mRNA sequence into the Basic Local Alignment Search Tool (BLAST) available for the dog-genome at the NCBI homepage.

Quantitative PCR

The HPRT and RPS19 were first tested for stability of expression in the pituitary gland, that earlier has been shown for other canine tissues.[13] The presence of tumorous tissue in the pituitary sample was analyzed with qPCR to POMC and GH expression. Only tissue samples with a high POMC to GH relation were included in the qPCR study (data not shown).

The expression of LIF in the pituitary was in general low. There was no detectable LIF in 4 out of 14 adenomas, 4 out of 12 normal pars distalis and in 1 out of 9 neurointermediate lobes. The highest values of normalized expression of LIF were measured in pituitary adenomas. However, the expression of LIF was not significantly different between the groups (Figure 7A).

The expression of LIFR normalized to Tbx19 expression was significantly higher in the normal pars distalis than that in the neurointermediate lobe and in the corticotroph adenomas (Figure 7B).

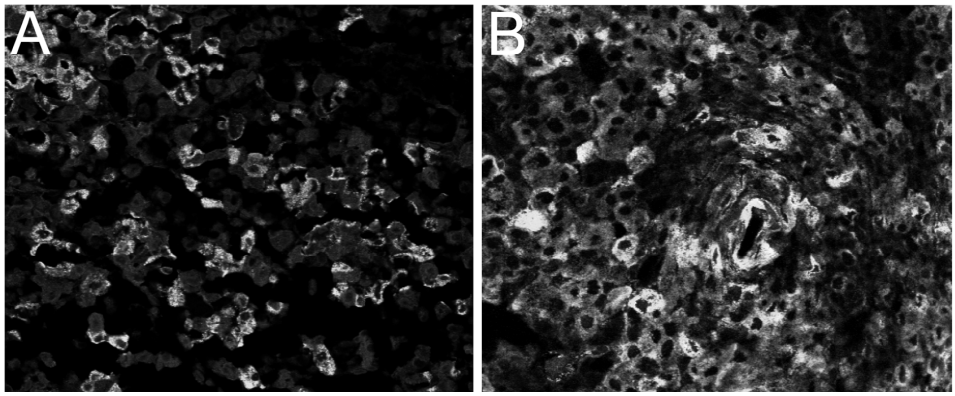

Figure 3. Immunofluorescence double-staining with leukemia inhibitory factor receptor α (LIFRα) (green) and adrenocorticotropic hormone (ACTH) (red) of the *pars distalis adenohypophysis* of a control dog (A) and a corticotroph adenoma (B) from a dog with pituitary-dependent hyperadrenocorticism (Cushing's disease). Co-localization gives a yellow staining (Magnification 800 x).

Discussion

This report shows that the expression of LIFRα is highly specific for POMC-expressing cells of the canine pituitary gland and corticotroph adenomas, and that a presence of a corticotroph adenoma is indicated by a nuclear immunoreactivity to LIFRα in non-tumorous cells of the pars distalis.

LIF

The expression of LIF was dispersed evenly throughout the pars distalis, and was only partly co-localized with ACTH, which is in agreement with previous findings on fetal pituitary glands (20 wk gestation) where LIF co-localizes with 30% of ACTH expressing cells, about 20% of GH expressing cells (somatotrophs) and about 15% of non-hormone expressing cells.[5] The expression of LIF was generally low in the pars distalis and surprisingly low, almost completely absent in the pars intermedia. The intensity of LIF staining varied considerably among the adenomas, of which a few showed a high expression

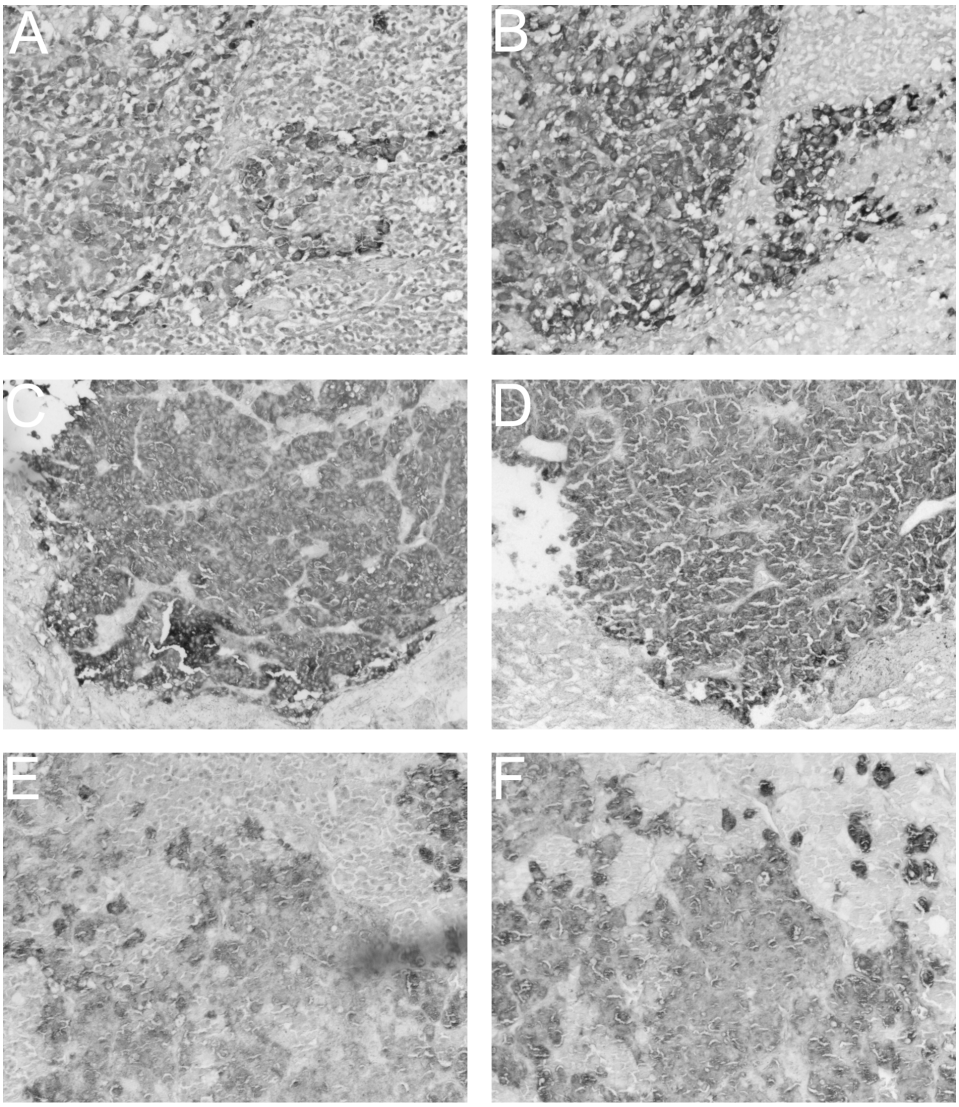

Figure 4. Immunoreactivity detected with DAB to leukemia inhibitory factor receptor (LIFRα) (left) and adrenocorticotropic hormone (ACTH) (right) in corticotroph adenomas from dogs with pituitary-dependent hyperadrenocorticism (Cushing's disease). The LIFRα co-localises with ACTH. In two specimens (A and E) an intranuclear immunoreactivity can be seen in the adjacent non-tumorous tissue (Magnification 400 x).

in comparison to normal tissue. The heterogenous immunoreactivity for LIF in the corticotroph adenomas is in agreement with previous findings on human corticotroph adenomas by Kontogeorgos and co-workers[28] In that series, 98 pituitary adenomas were investigated including 31 corticotroph adenomas. Of these, only 24 (77.5%) were

immunopositive for LIF protein. However, the staining grade of the corticotroph adenomas was the lowest among the different types of pituitary adenomas.

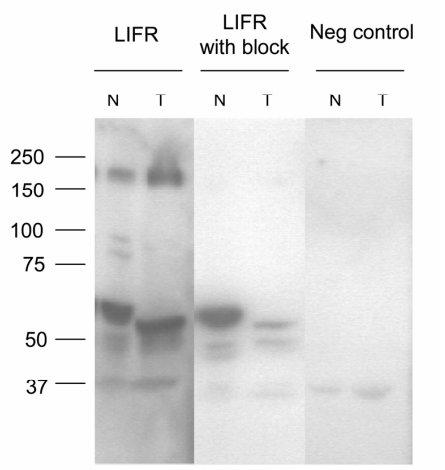

Figure 5. Protein-blot analysis using antibodies directed to leukemia inhibitory factor receptor (LIFRα) (left). N = *pars distalis adenohypophysis* from a control dog; T=corticotroph adenoma (T). The antibody reactivity was blocked by preincubation with a specific blocking peptide (LIFR with block), and the LIFR antibody was omitted as a negative control (Neg control). The expected size of LIFRα was 190 kDa. Additional two smaller proteins, 80 and 90 kDa, can be seen in the control sample. A smaller, partially blocked, protein is seen at about 50 kDa in the adenoma tissue.

LIFR

The co-expression of LIFR and ACTH seen in the present study is in agreement with the indirect findings made by Akita and co-workers 1995, who sorted pituitary cells by the use of an anti-LIF antibody. These sorted cells consisted almost exclusively of ACTH-secreting cells.[5] However, to the author's knowledge, this is the first time that a direct immunostaining for LIFR has been performed on pituitary tissue in any species.

An explanation for the strong immunoreactivity of LIFR in the intermediate lobe despite an almost complete absence of LIF-expression may be that the cells secrete another cytokine of the IL-6 family e.g., oncostatin M, that make use of the LIFR for signalling transmission. However, the predominant cell type in the intermediate lobe is the melanotroph. The melanotrophs secrete α-MSH,[15] which is a strong anti-inflammatory agent and regulator of the early-phase hypothalamic response to cytokine stimulation.[17,47] Regarding the rich arterial blood supply to the neurohypophysis and the longitudinal vessels running in close apposition to the intermediate lobe, it may be hypothesized that the expression of LIFR on the melanotrophs enables these cells to react on circulating cytokines and thereby take part in a fast-reacting anti-inflammatory response system.[17]

It is well-known that the LIF-LIFR signalling cascade, intracellularly mediated by the Jak-STAT pathways, promotes ACTH expression.[2,3,5,10,42,43,46,48] The distal promoter region of ACTH contains a STAT-binding-site in close vicinity to the Nur-responsive element[30] Down-regulation of the LIFR in normal corticotrophs from pituitaries harbouring a corticotroph adenoma, may therefore contribute to the reduced ACTH expression in these cells. Interestingly, there was a nuclear/perinuclear LIFR-immunoreactivity in non-neoplastic cells of the pars distalis in pituitaries harbouring a corticotroph adenoma. This reactivity disappeared with the use of a specific blocking peptide. This may indicate the presence of a tumor-dependent transformation of the non-tumorous cells of the pars distalis. Also, nuclear localization has been documented for another receptor of the cytokine family, the prolactin

receptor. In this case, the wild-type hPRLr is translocated to the nucleus where it potentiated STAT5a transactivation.[20]

The LIFR is a complex molecule with an intricate regulation of gene transcription. There are two forms of LIFR recognized in human and mouse; a membrane-bound form (LIFRα), and a soluble form (sLIFR). The soluble form lacks the transmembrane and cytoplasmic part of the receptor and is secreted into the circulation where it binds LIF and thereby acts as an antagonist.[31,32,39,52,57] In addition at least two promoter regions have been identified in human cells.[6,54] Our data from the protein-blot assay showed LIFR-immunoreactive proteins of different lengths. A short form of LIFR in the region 50-60 kDa was detected in a pituitary gland tumor. Short forms of LIFR-immunoreactive proteins have previously been reported in

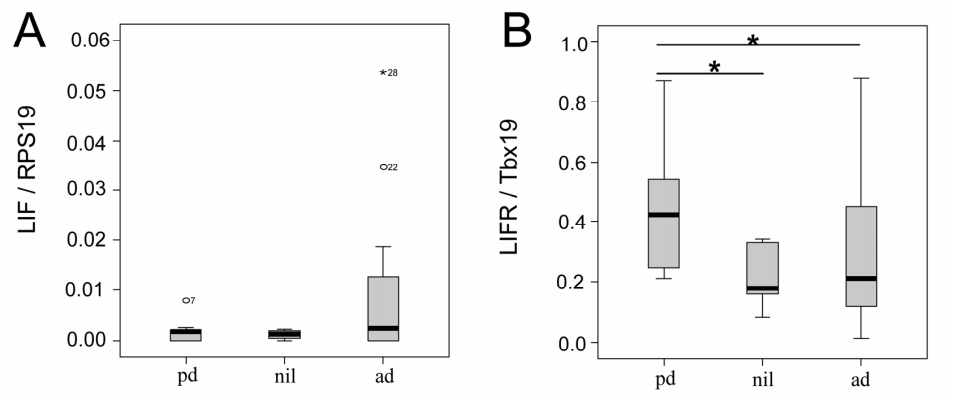

Figure 6. Box-plot graphs representing the expression of leukaemia inhibitory factor (LIF) normalized by the reference gene ribosomal protein S19 (RPS19) (A) and the expression of the LIF receptor normalized by the corticotroph and melanotroph-specific T-box transcription factor Tbx19 (B). The boxes represents expression in the *pars distalis adenohypophysis* (pd), neurointermediate lobe (nil), and canine corticotroph adenomas (ad).

studies of hepatic cells and testicular tissue [8,19]. The interpretations were different. In the hepatic cells, short proteins immunoreactive to LIFRα were interpreted as LIFR degradation products, which increased with cytokine stimulation concurrently with a decrease in the presence of full-length LIFR.[8] If this is the case, presence of a short form of LIFR in corticotroph adenomas indicate the presence of cytokine stimulation. However, no reduction in full-length LIFR was seen. Taken together these findings may suggest that there is a loss of function in the inhibiting mechanism of the expression of LIFRα. Another possibility, in agreement with the interpretation of the findings in the testis,[19] is that there may be a specific expression of a truncated form of LIFRα in parallel to what has been found for the human prolactin receptor.[20]

The expression of LIFR was generally high in both normal and adenomatous tissue, which is in agreement with previous findings.[55] However, when normalized to the expression the corticotroph-specific Tbx19, the expression of LIFR was significantly higher in the pars distalis than in the pars intermedia or adenomas. The mutation-analysis of pituitary tissue from 14 pituitary adenomas revealed no mutations which is in agreement with what has been reported for human pituitary adenomas.[26]

To the author's knowledge, the present report is the first of its kind to present immunoreactivity data for pituitary tissue using antibodies directed to LIFR. There was a striking co-localization between LIFR and the POMC. It has been recognized that STAT-signalling pathways are important for tumor biology.[27] Retinoic acid inhibits LIF-signalling pathways in mouse embryonic stem cells by down-regulation of LIFR[51] and by interaction with the nuclear receptors Nur77 and Nurr1.[40] Recently, it has been presented that retinoic acid reduces plasma concentrations of ACTH in dogs with PDH [14]. Therefore, taken together, our findings and those of previous studies indicate that LIFR may play an important role in the pathogenesis of corticotroph adenomas in the dog.

It can be concluded that it is unlikely that a mutated LIFR plays an important role in the pathogenesis of corticotroph adenomas in the dog. However, there is a strong co-expression of LIFR and POMC in the canine pituitary and activation of the LIFR pathway may still be important of the tumor formation.

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