Effects of corticotrophin-releasing hormone, vasopressin and insulin-like growth factor-I on proliferation of and adrenocorticotrophic hormone secretion by canine corticotrophic adenoma cells *in vitro*

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Abstract

Extrinsic factors such as hypothalamic hormones or intrapituitary growth factors may stimulate clonal expansion of a genomically altered cell and therefore play a role in pituitary tumorigenesis. Here we report on the effects of the hypophysiotrophic hormones corticotrophin-releasing hormone (CRH) and vasopressin (AVP) and the intrapituitary growth factor insulin-like growth factor-I (IGF-I) on the proliferation of, as measured by the bromodeoxyuridine labelling index, and ACTH secretion by normal canine pituitary cells and corticotrophic adenoma cells of dogs with pituitary-dependent hyperadrenocorticism. The sensitivity to inhibition by cortisol was analysed under various conditions.

Under basal conditions, no significant differences were found in the bromodeoxyuridine labelling indices between control cells and tumour cells. CRH, AVP, IGF-I and cortisol had no effect on the proliferation of canine pituitary cells or canine corticotrophic adenoma cells. In contrast with normal pituitary cells, the proliferation of corticotrophic adenoma cells was stimulated by fetal calf serum (FCS). This FCS-induced proliferation was not inhibited by cortisol.

The CRH-induced ACTH secretion by corticotrophic adenoma cells was significantly ($P < 0.05$) lower than that by normal pituitary cells after 4 h incubation with CRH. Incubation with cortisol for 24 h resulted in reduced ACTH secretion under basal and AVP- or IGF-I-stimulated conditions. The relative inhibition was, however, significantly ($P < 0.05$) lower in ACTH-producing tumour cells than in normal pituitary cells. Cortisol did not inhibit the CRH-induced ACTH secretion in normal pituitary cells after 24 h.

In conclusion, canine corticotrophic adenomas are less sensitive to stimulation by CRH and less sensitive to inhibition by glucocorticoids. These tumours have an aberrant sensitivity to a growth-promoting factor present in FCS. This factor may have an important role in the growth promotion of canine corticotrophic tumours.

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Introduction

Pituitary adenomas are currently believed to be monoclonal in origin, indicating that they arise from a genetically transformed cell. The promotion of this transformed cell can be caused by intrinsic mutations and/or by extrinsic factors such as hypothalamic hormones or intrapituitary growth factors.

Activating mutations in the α -subunit of the Gs protein have been found in about 40% of the growth hormone-secreting adenomas (1, 2), in 10% of non-functioning pituitary adenomas (3) and in 6% of corticotrophic adenomas (4). In a small number of pituitary carcinomas, mutations in the retinoblastoma (5), H-ras (6, 7) and protein kinase C (8) genes have been reported.

These changes have been found in relatively few tumours (9, 10) and there is as yet no evidence that other cellular oncogenes or tumour suppressor genes play a role in the development of pituitary adenomas.

The clonal expansion may require the action of hypothalamic hormones and/or intrapituitary growth factors. Administration of corticotrophin-releasing hormone (CRH) and vasopressin (AVP) to rats increases the number of corticotrophic cells (11–14). Growth hormone-releasing hormone transgenic mice develop somatotrophic adenomas (15) and gonadotrophin-releasing hormone stimulates gonadotrophic cell proliferation in rats (16). Moreover, the pituitary gland contains several growth factors (17, 18), such as insulin-like growth factor (IGF-I/II) (19), epidermal

growth factor (20, 21), basic fibroblast growth factor (22–24) and transforming growth factor- α/β (18). These growth factors may affect the function and proliferation of pituitary cells through autocrine or paracrine actions in the pituitary (25). We have shown stimulating effects of CRH, AVP, basic fibroblast growth factor, epidermal growth factor and IGF-I on the proliferation of a murine corticotrophic tumour cell line (AtT20) (26). The growth factor-induced proliferation of AtT20 cells appeared to be less sensitive to inhibition by glucocorticoids than the proliferation induced by hypothalamic hormones, and the IGF-I-induced proliferation was the least sensitive to inhibition by cortisol (26).

Here we report on the effects of CRH, AVP and IGF-I on the proliferation of and adrenocorticotrophin (ACTH) secretion by normal pituitary cells and corticotrophic adenoma cells of dogs with pituitary-dependent hyperadrenocorticism (PDH), which is generally regarded to be a valuable model for pathogenetic studies (27). The sensitivity to inhibition by cortisol was analysed under various conditions.

Materials and methods

Tissue culture

Pituitaries were collected by trans-sphenoidal hypophysectomy in 12 clinically healthy dogs and 8 dogs with PDH. Diagnosis of PDH was based upon measurements of corticoid/creatinine ratios in urine before and after dexamethasone administration (28), measurements of plasma ACTH concentration (29) and computed tomography of the pituitary gland (30). Total hypophysectomy was performed by the trans-sphenoidal approach described by Markowitz *et al.* (31), with slight modifications. Anaesthesia was induced by the administration of 0.015 mg fentanyl/kg, 0.75 mg droperidol/kg (Thalamonal; Janssen Pharmaceutica BV, Tilburg, The Netherlands) and 0.05 mg atropine/kg (Atropini Sulfas; Pharmachemie BV, Haarlem, The Netherlands), followed by the i.v. administration of 5 mg propofol/kg (Diprivan; Zeneca BV, Ridderkerk, The Netherlands). The trachea was intubated and anaesthesia was then maintained by inhalation of a mixture of isoflurane (Forene; Abbott BV, Maarssen, The Netherlands), NO₂ and oxygen in a semi-closed system. Analgesia was provided by sufentanil (Sufenta; Janssen Pharmaceutica BV) administered by an i.v. pump at a rate of 2.5 μ g/kg per h.

Immediately after its removal, pituitary tissue was placed in Hanks balanced salt solution (HBSS) (JRH Biosciences, Lenexa, KS, USA) supplemented with 2 mM L-glutamine (Biochrom KG, Berlin, Germany), 10 U penicillin/ml (Kombivet, Etten-Leur, The Netherlands) and 10 mg streptomycin/ml (Alfasan, Woerden, The Netherlands) at 4°C. The pituitary fragments were washed three times with HBSS, minced and incubated in dispase (2.4 U/ml HBSS) (Boehringer, Mannheim,

Germany) for 90–120 min at 37°C. After two washes with HBSS, the cells were mechanically dispersed with a tissue grinder. The dispersed cells were plated on to 24-well plates (Greiner, Alphen a/d Rijn, The Netherlands) and grown at 37°C in a humidified atmosphere of 5% CO₂/95% air. The culture medium consisted of minimum essential medium supplemented with D-valine (Life Technologies, Breda, The Netherlands), 10% fetal calf serum (FCS) (Sebak, Aidenbach, Germany), 10 U penicillin/ml and 10 mg streptomycin/ml.

Proliferation studies

During the proliferation studies, the FCS in the medium described above was replaced by growth factor-inactivated FCS (SH-FCS) supplemented with 0.2% (w/v) BSA (Sigma, St Louis, MO, USA), 30 nM Na₂SeO₃ (Sigma) and 10 μ g transferrin/ml (Sigma) (32).

The proliferation studies were conducted with tumour material from seven dogs with PDH and six pituitaries from clinically healthy dogs. The cells were plated on to poly-D-lysine-coated coverslips (Nunc Inc, Naperville, IL, USA) at a density of 2×10^4 cells per well and were grown for 2 days in medium with 10% FCS. The cells were washed and incubated with SH-FCS medium supplemented with 10 nM CRH (Peninsula Laboratories, St Helens, Merseyside, UK), 100 nM AVP (Sigma), 100 ng IGF-I/ml (Boehringer) or medium with 10% FCS, with and without 100 nM cortisol (Sigma) (three wells per incubation) at day 0. On day 4 of incubation, the medium was changed. On day 7 of incubation, the cells were incubated with bromodeoxyuridine (BrdU; 20 nmol/ml per well; following the protocol described in the 5-bromo-2-deoxyuridine labelling and detection kit II from Boehringer) for 1 h. The cells were fixed with ethanol and incubated with mouse anti-BrdU. After incubation with anti-mouse IgG-alkaline phosphatase followed by the substrate reaction, bound anti-BrdU was visualized by light microscopy.

Secretion studies

The secretion studies were conducted with tumour material from eight dogs with PDH and 12 pituitaries obtained from clinically healthy dogs. Cells were plated on to 24-well plates at a density of 10^5 cells per well in medium with FCS, as described above. After 2 days, the cells were washed and incubated with medium in which FCS was replaced by 0.2% (w/v) BSA and supplemented with 10 nM CRH, 100 nM AVP or 100 ng IGF-I/ml, with and without 100 nM cortisol. After 4 and 24 h, the medium was removed from the cells and stored at –20°C. The ACTH content of the medium was measured by RIA without extraction, by the procedure of Arts *et al.* (33). Antiserum was obtained from IgG Corporation (Nashville, TN, USA). The tracer was purchased from International CIS (St Quentin-Yvelines,

Figure 1 BrdU labelling indices (BrdU LI) of normal canine pituitary cells and canine corticotrophic tumour cells after 7 days of incubation with CRH (10 nM), AVP (100 nM), IGF-I (100 ng/ml) or 10% FCS with or without 100 nM cortisol. Data are expressed as mean $\pm$ S.E.M.. ♦, BrdU LI is significantly higher than in SH-FCS medium without cortisol. $P < 0.05$ was considered significant.

France), and the standard was obtained from the National Institutes of Health (Bethesda, MD, USA). The detection limit was 10 ng/l. Interassay variation was 12%.

Calculations and statistics

To determine the BrdU labelling index, at least 250 cells/coverslip (three coverslips/incubation) were counted. The BrdU labelling index was calculated as the percentage of the total number of nuclei counted that were BrdU labelled. The results are presented as mean $\pm$ S.E.M.

In the secretion studies, the average ACTH concentration per incubation (three wells/incubation) was determined and expressed as a percentage of ACTH secretion into the control medium. The percentage change in ACTH secretion induced by cortisol was calculated per incubation. The results are presented as mean $\pm$ S.E.M.

Differences between the incubations were tested, using the SPSS statistical software package, with the Friedman test followed by the Wilcoxon signed ranks test for differences within groups. Differences between groups were calculated using the Mann-Whitney test. $P < 0.05$ was considered significant.

Results

In the normal pituitary, the corticotrophic cells constitute about 10% of the adenohypophyseal cells (34), whereas in corticotrophic adenomas this percentage is far exceeded. The absolute amount of ACTH production differs between primary cultures of adenoma and normal pituitary cells. These differences hamper the

comparison of the absolute effects of various factors on the proliferation of and ACTH secretion by corticotrophic cells. Therefore, in the proliferation studies, values are expressed as percentage BrdU-positive cells and in the secretion studies values are expressed as percentage of the control (the absolute values are set at 100%).

Proliferation studies

After incubation in the growth-factor depleted medium (SH-FCS medium), the differences in BrdU labelling index between the control cells ($0.9 \pm 0.3\%$; $n = 6$) and the tumour cells ($0.5 \pm 0.2\%$; $n = 7$) were not significant. The hypophysiotrophic hormones CRH and AVP, the intrapituitary growth factor IGF-I, and cortisol had no effect on the proliferation of canine pituitary cells or canine corticotrophic adenoma cells (Fig. 1). In the tumour cells, incubation with FCS ($4.0 \pm 1.4\%$) resulted in significantly ($P < 0.05$) higher BrdU labelling index compared with incubation with SH-FCS medium, whereas in the control cells, the BrdU labelling index after incubation with FCS ($1.3 \pm 0.4\%$) was not different from that after incubation with SH-FCS medium. FCS-induced proliferation of the corticotrophic adenoma cells was not inhibited by cortisol ($3.2 \pm 0.8\%$).

Secretion studies

Basal ACTH secretion by normal pituitary cells ($n = 12$) amounted to 746 ± 156 and 2800 ± 413 ng/l after 4 and 24 h of incubation respectively. ACTH secretion increased significantly ($P < 0.05$) after 4 and 24 h of incubation with CRH ($316.8 \pm 39.8\%$ and $236.2 \pm 22.1\%$), AVP ($123.3 \pm 8.8\%$ and $113.5 \pm 5.1\%$) and IGF-I ($136.4 \pm 13.2\%$ and $137.2 \pm 13.8\%$) compared

with ACTH secretion in the control medium (100%). Cortisol significantly ($P < 0.05$) inhibited the ACTH secretion stimulated by CRH ($28.1 \pm 4.3\%$), AVP ($8.9 \pm 4.2\%$), and IGF-I ($10.0 \pm 2.0\%$) after 4 h of incubation. After 24 h of incubation, cortisol significantly ($P < 0.05$) inhibited basal ACTH secretion ($53.9 \pm 5.6\%$) and the ACTH secretion stimulated by AVP ($56.6 \pm 5.1\%$) and IGF-I ($66.2 \pm 3.3\%$), whereas the CRH-induced ACTH secretion was not significantly inhibited ($3.1 \pm 6.7\%$) (Figs 2a and b).

The basal ACTH secretion by corticotrophic adenoma cells ($n = 8$) amounted to 2600 ± 1377 and 3028 ± 1555 ng/l after 4 and 24 h incubation respectively. The ACTH secretion was significantly stimulated by CRH after 4 h ($184.2 \pm 19.6\%$) and 24 h ($309.7 \pm 44.1\%$) as well as by AVP ($142.7 \pm 14.0\%$) after 24 h. The CRH-induced ACTH secretion was significantly ($P < 0.05$)

inhibited by cortisol ($26.5 \pm 5.5\%$ and $20.0 \pm 7.7\%$) after 4 and 24 h of incubation. Cortisol significantly ($P < 0.05$) inhibited basal ACTH secretion ($21.9 \pm 6.1\%$) and the ACTH secretion stimulated by AVP ($24.1 \pm 9.7\%$) after 24 h of incubation. The ACTH secretion by corticotrophic adenoma cells was not stimulated by IGF-I after 4 and 24 h of incubation. During co-incubation with IGF-I, basal ACTH secretion was not inhibited by cortisol after 4 and 24 h (Figs 2c and d).

After 4 h of incubation, the CRH-induced ACTH secretion by canine corticotrophic tumour cells was significantly ($P < 0.05$) lower than that by normal canine pituitary cells.

During the 24 h incubations, the inhibition by cortisol of basal ACTH secretion and the inhibition by cortisol of the ACTH secretion during co-incubation

Figure 2 ACTH concentrations in media from normal canine pituitary cells (a and b) and canine corticotrophic tumour cells (c and d) after 4 and 24 h of incubation with CRH (10 nM), AVP (100 nM) or IGF-I (100 ng/ml) with or without 100 nM cortisol. ACTH concentration is expressed as a percentage of that in the control medium. Data are expressed as mean $\pm$ S.E.M. ♦, Statistically different from the ACTH concentration in the control medium. ◇, Significant inhibition by cortisol. $P < 0.05$ was considered significant.

with AVP and IGF-I were significantly ($P < 0.05$) lower in ACTH-producing tumour cells than in the same incubations with normal pituitary cells.

Discussion

Of the hypothalamic factors that regulate ACTH secretion from the anterior pituitary, CRH and AVP are the most potent stimulatory neurohormones (35–37). After transformation of pituitary corticotropes into ACTH-producing adenomas, the responsiveness to CRH and AVP is altered and there is a decreased sensitivity to feedback by glucocorticoids. Apart from their effect on the release of ACTH, hypothalamic hormones and intrapituitary growth factors may facilitate clonal expansion of the genetically altered corticotrophic cell.

In the present study, the hypothalamic hormones CRH and AVP were found to have no stimulatory effects on the proliferation of normal canine pituitary cells nor on the proliferation of canine corticotrophic tumour cells. This indicates that hypophysiotrophic hormones do not play an important role in the promotion of ACTH-secreting corticotrophic adenomas in dogs with PDH (Cushing's disease). This is in concordance with results from earlier studies. In man and dog, it has been shown that the excessive ACTH secretion in Cushing's disease is not caused by persistent hyperstimulation of corticotropes by CRH (29, 38), and canine ACTH-producing tumours have been shown to be relatively insensitive to stimulation by hypothalamic hormones *in vitro* (39).

The anterior pituitary gland consists of cells with a relatively low mitotic rate. The proliferative activity of canine hypophyseal cells *in vivo* is 0.05% (40), which is lower than the proliferation rate *in vitro*. We found FCS to have a stimulating effect on the proliferation of canine corticotrophic adenoma cells *in vitro*. This indicates that canine ACTH-producing pituitary tumour cells develop an aberrant sensitivity to growth-promoting stimulation by a factor present in FCS. It would be very interesting to find out what factor is responsible in order to know which signal-transduction pathway becomes activated in these (post-mitotic) cells. This factor, and therefore the signal-transduction pathway activated, may have a profound role in the clonal expansion of these tumours. From the results of this study, we conclude that the factor is not CRH, AVP or IGF-I. For preparation of the growth factor-depleted serum, FCS was treated with dithiothreitol and iodoacetamide, which destroys disulphide bridges, and dialysed for 3–4 days (molecular mass cut-off 6–8 kDa). Polypeptide growth factors require intact disulphide bridges for their activity (32). The unknown factor in FCS may therefore be a factor containing disulphide bridges, such as polypeptide growth factors, and/or be a factor with a molecular mass below 6–8 kDa. The idea that the unknown factor may be a growth factor is supported by results from our study with AtT20 cells. Proliferation of murine corticotrophic

tumour cells (AtT20) induced by growth factors is, in comparison with hypothalamic neurohormones, relatively insensitive to inhibition by glucocorticoids (26). The FCS-induced proliferation of canine corticotrophic tumour cells is not sensitive to inhibition by glucocorticoids.

Because the amount of cells harvested was not sufficient to test a great variety of growth factors, we decided, on the basis of our study with AtT20 cells (26), to test CRH, AVP and IGF-I alone and in combination with cortisol. As none of these peptides mimic the FCS-induced proliferation, further investigations are needed to elucidate the stimulating factor present in FCS. Several candidate peptides can be put forward. Tilemans *et al.* (41) showed that the development of prolactin-, growth-hormone- and ACTH-containing cells in the anterior pituitary of the rat is modulated by luteinizing hormone-releasing hormone and that the action of this hormone is mediated by specific growth factors released from gonadotropes. These specific growth factors were not identified in this study. Mitogenic effects of epidermal growth factor on rat corticotropes have been reported by Childs *et al.* (14), albeit in a limited dose range. A paracrine or autocrine role for this growth factor in corticotrophic function has been postulated (42, 43). Pituitary adenoma cells have been reported to express membrane-anchored transforming growth factor- α , indicating a role for it in pituitary tumorigenesis (44).

We have also shown that canine corticotrophic adenoma cells are less responsive to stimulation by CRH than are normal canine pituitary cells. These results confirm earlier reports on *in vitro* and *in vivo* studies (39, 45) that demonstrated a relative insensitivity of canine corticotrophic tumour cells to stimulation by CRH.

CRH-induced ACTH secretion from normal canine pituitary cells and from canine corticotrophic tumour cells was inhibited by glucocorticoids after 4 but not 24 h of incubation. The inhibition found after 4 h can be explained by the rapid inhibitory effect of glucocorticoids involving inhibition of the CRH-induced secretory process. To understand the observation that CRH-induced ACTH secretion could not be inhibited after 24 h, two phenomena have to be considered. First, for human corticotrophic adenoma cells it has been reported that CRH is able to down-regulate glucocorticoid receptor mRNA, which may lead to a decreased response to glucocorticoids (46). Secondly, CRH-induced ACTH secretion has been reported to be rapidly desensitized in rat anterior pituitary cells when cultured in the absence of added glucocorticoids (47). The response of corticotrophic cells to CRH *in vitro*, cultured in the absence of glucocorticoids, would thus be relatively low compared with the *in vivo* situation in which background levels of glucocorticoids are present.

The impaired inhibition by glucocorticoids of the basal ACTH secretion of corticotrophic tumour cells during incubation with IGF-I seems to result from the

lack of sensitivity of corticotrophic tumour cells to glucocorticoid inhibition, rather than to the ability of IGF-I to prevent glucocorticoid-provoked inhibition of ACTH secretion, as has been described for murine corticotrophic tumour cells (26).

In conclusion, the hypothalamic hormones CRH and AVP and the intrapituitary growth factor IGF-I do not have growth-stimulating effects on canine corticotrophic tumour cells. Canine corticotrophic tumours have an aberrant sensitivity to a growth-promoting factor present in FCS. This factor may have a profound role in the clonal expansion of these tumours and, in combination with the genetic alterations of the corticotrophic tumour cells, be responsible for the tumorigenesis of canine corticotrophic adenomas.

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