Retinoic Acid as a Novel Medical Therapy for Cushing’s Disease in Dogs

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Cushing’s disease is almost always caused by an ACTH-secreting pituitary tumor, but effective medical therapy is currently limited. Because retinoic acid has been shown to be potentially useful in decreasing corticotroph secretion and proliferation in rodent models, we have studied its action in dogs with Cushing’s disease. A randomized treatment with retinoic acid (n = 22) vs. ketoconazole (n = 20) in dogs with Cushing’s disease was assigned for a period of 180 d. Clinical signs, plasma ACTH and α-MSH, the cortisol/creatinine urine ratio, and pituitary magnetic resonance imaging were assessed and compared at different time points. We recorded a significant reduction in plasma ACTH and α-MSH, and also in the cortisol/creatinine urine ratio, of the dogs treated with retinoic acid.

Pituitary adenoma size was also significantly reduced at the end of retinoic acid treatment. Survival time and all the clinical signs evaluated showed an improvement in the retinoic-acid-treated dogs. No adverse events or signs of hepatotoxicity were observed, suggesting that the drug is not only effective but also safe. Retinoic acid treatment controls ACTH and cortisol hyperactivity and tumor size in dogs with ACTH-secreting tumors, leading to resolution of the clinical phenotype. This study highlights the possibility of using retinoic acid as a novel therapy in the treatment of ACTH-secreting tumors in humans with Cushing’s disease. (Endocrinology 147: 4438–4444, 2006)

PITUITARY TUMORS COMPRISE some 10% of intracranial neoplasms, and many of these are secretory. Although the primary therapy for nonsecretory adenomas is surgical, many of the secreting tumors may now be managed, as primary or secondary treatment, with medical therapy. Thus, the majority of prolactin-secreting tumors, prolactinomas, may be treated with dopamine agonist drugs such as cabergoline (1), whereas GH-secreting tumors will frequently respond to somatostatin analog therapy, both in terms of hormone secretion as well as tumor shrinkage (2, 3). However, for ACTH-secreting pituitary tumors causing pituitary-dependent Cushing’s syndrome, Cushing’s disease, only a minority respond to treatment with dopamine agonists or somatostatin analogs (4–6), and the primary mode of therapy remains transsphenoidal surgery. Such corticotroph cell adenomas, or corticotrophinomas, account for approximately 8% of all clinically recognized pituitary adenomas, possibly more when considering silent corticotroph adenomas (7, 8). The glucocorticoid hypersecretion secondary to Cushing’s disease causes significant morbidity, but unfortunately transsphenoidal surgery is associated with only a 60–80% cure rate for microadenomas, even in the most experienced hands, as well as a significant rate of recurrence; the cure rate is even less for macroadenomas. In addition, bilateral adrenalectomy can give rise to progression of the pituitary tumor, Nelson’s syndrome, in a large minority of patients (9–12).

Some pharmacological agents have been proposed for the medical treatment of Cushing’s disease (5, 6, 13, 14). Octreotide, a somatostatin analog, and dopaminergic and serotonergic drugs have been used to control ACTH secretion and tumor growth (5), but they have shown no consistently effective results in a large number of patients for extended periods. Several adrenal target drugs, such as ketoconazole, have also been used (5, 13, 14), but although they control the excessive glucocorticoid secretion in some patients, they do not inhibit corticotrophinoma growth and its consequent effects and are not a real alternative to the established procedures.

Ligands for the nuclear receptor, peroxisome proliferator-activated receptor-γ (PPAR-γ), induce cell-cycle arrest and apoptosis in corticotrophinoma cells, induced tumor growth arrest in vivo in a nude mouse model, and inhibited ACTH and corticosterone secretion from tumor cells (15) but seem not to be effective in humans according to small-scale clinical trials (16). Retinoic acid is an agent that has been shown to
inhibit proliferation, invasion, and tumor growth in vitro and induces differentiation and apoptosis in different cell types (17–19). Some of these effects are mediated by a reduction in binding of the transcription factors AP-1 and Nur77 to their cognate DNA sites (20, 21); these factors are also essential in the control of the proopiomelanocortin (POMC) gene (22–24), which gives rise to the precursor to ACTH and α-MSH. Very recently, we have shown that retinoic acid inhibits ACTH secretion both in vitro and in vivo through an action on POMC gene transcription and also inhibits corticotrophinoma development and proliferation (25).

Recently, the genome sequence of the domestic dog has been published, these data providing considerable information as to the evolution of this species. However, in addition, because of the similarity of canine to many human diseases, it has been suggested that the dog may help to bridge the gap between preclinical drug studies and the effects of the same drug in humans (26). Cushing’s disease is a common disorder in dogs and one in which the histopathology is broadly similar to that in the human and for which the treatment is also primarily surgical where available (27). We therefore speculated that retinoic acid may be useful therapeutically in the treatment of canine Cushing’s disease and have assessed its use in this species as a specific treatment modality and as an initial model system for the human (26). Our results suggest that retinoic acid may indeed be a potent medical treatment for Cushing’s disease, and human trials are now warranted.

**Materials and Methods**

**Population under study**

We selected 42 dogs with Cushing’s disease (15 males) seen sequentially according to the following criteria: the presence of at least four clinical signs characteristic of the disease, urinary values for the cortisol/mol/liter:creatinine ratio greater than 70 mol/liter, a detectable plasma ACTH, and evidence of a pituitary adenoma on magnetic resonance imaging (MRI). These criteria are considered pathognomonic of an anterior lobe corticotrophinoma (27). The animals were studied at the Endocrinology Unit of the School Hospital of the Faculty of Veterinary Sciences, University of Buenos Aires, Argentina. The average age of the animals at the time of diagnosis was 9 yr (95% confidence limits, 8.2–9.9 yr; range, 3–14 yr) without significant difference between males and females. At the time of diagnosis, no animal presented any concurrent disease other than that associated with Cushing’s disease, which included elevations in serum transaminases glutamate-pyruvate transaminase (GTP) and glutamyl oxaloacetic transaminase (GOT) and alkaline phosphatase (ALP) previously described in dogs with Cushing’s disease (27).

All dogs seen and following the above stated criteria were consecutively included in the study and were alternatively randomly distributed into two groups: 1) a retinoic-acid-treated (Rx) group of 22 dogs (seven males and 15 females, of which 12 were not castrated and were anestrous for at least 18 months and three were castrated) were treated with 2 mg/kg body weight/d of 9-cis retinoic acid (Roche, Basel, Switzerland); and 2) a ketoconazole-treated (Ktz) group (control) of 20 dogs (eight males and 12 females, none of which were castrated and all were anestrous for >18 months) were treated with 20 mg/kg body weight/d ketoconazole (Jensen Laboratories, Buenos Aires, Argentina). Ketoconazole is a drug that interferes with steroid biosynthesis and is a standard therapy for canine Cushing’s disease (28); it was used for comparison because of ethical and legal reasons that would not allow us to leave animals without an already proven treatment. The dogs comprised a variety of breeds, but these were randomly allocated between the two groups; 9-cis retinoic acid was chosen because it binds both retinoic acid receptor and retinoid X receptor (RXR) (18, 29). The dose of ketoconazole was based on current therapeutic custom, whereas the retinoic acid dosage was based on current clinical therapy in the human and the results of pilot studies.

**Study protocol**

Drugs were administered for 180 d. During this period, hepatic enzyme activity (ALP, GTP, and GOT) was measured by a kinetic automated method (Metrolab, Merck Autoanalyzer; Merck, Darmstadt, Germany) every 30 d; an increase of more than three times the ALP and GTP or of two times the GOT values found at the time of diagnosis was considered to indicate hepatocyte necrosis and as a reason for suspension of treatment, but in no animal was this threshold breached. None of the animals treated with any of the drugs showed any clinical signs of hepatic dysfunction during or after the treatment.

**Clinical signs**

The following clinical signs, considered critical in dog Cushing’s disease (27), were monitored by a single observer. 1) Fluid ingestion and micturition were evaluated by means of measurement of daily intake of water (100 ml/kg-d maximum); polydipsia and polyuria were present in 88% of the cases before treatment. 2) Solid ingestion, assessed by the frequency of food intake (more than three daily meals), was monitored. Hyperphagia was initially present in 81% of the cases. 3) Estrous cyclicity, in terms of a return of the cycle in those females who were not castrated, was recorded. 4) Dermatological signs such as the elasticity and thickness of the skin and the presence of striae were recorded as well as partial or total loss of body hair and its oiliness and brightness. Such dermatological signs were present in 76% of the dogs diagnosed with Cushing’s disease. 5) Abdominal swelling, either normal or a ball form, was recorded. The ball form was present in 93% of dogs before treatment. 6) An increase in weight was noted by 95% of dog owners before treatment. Given the variability in dog size, weight changes after treatment are expressed as percent change with respect to the weight at the beginning.

**Biochemical and endocrine studies**

For the cortisol/creatinine (RC/C) urine ratio, the methodology used was previously described by Rijnberk and colleagues (30) and modified in the Faculty of Veterinary Sciences, University of Buenos Aires. Representative 24-h urine samples were collected (an aliquot of 2 ml for each) as follows: the second voided urine in the morning, all urines during the rest of the day and the last one at night, and the first urine of the following day. Urinary cortisol was measured by a commercial RIA using solid-phase technology (DPC Corp., San Diego, CA) and creatinine by an automated kinetic method according to the manufacturer’s instructions (Metrolab Autoanalyzer; Merck). The intra- and interassay coefficients of variation were 8 and 5%, respectively, and the sensitivity was 1 nmol/liter. Evaluation times were at time 0 (at diagnosis), at 120 d (in treatment), and at 180 d (end of treatment).

Plasma ACTH and α-MSH were assayed on samples taken between 1300 and 1400 h because the dog shows circadian rhythmicity with a marked increase in plasma ACTH at this time of the day (31). ACTH (Nichols Advantage ACTH Assay; Nichols Institute Diagnostics, San-Vilbel, Germany) and α-MSH (Euro-Diagnostica AB, Malmö, Sweden) were measured by RIA as previously described (32). Both assays are established procedures in veterinary medicine (33). Both hormones were evaluated monthly from time 0 (diagnosis) to 180 d/6 months (end of treatment). The intraassay coefficients of variation for the ACTH immunoassay were 3 and 3.2% for mean values of 35 or 366 pg/ml, respectively, with interassay coefficients of variation of 7.8 and 6.8% for mean values of 36 or 358 pg/ml, respectively. The intraassay coefficients of variation for α-MSH were 11.8, 4.7, and 2.9% for mean values of 6.2, 33.6, and 77.7 pmol/liter, respectively, with interassay values of 13.0, 8.4, and 4.0% for α-MSH at 16.5, 37.8, and 79.6 pmol/liter, respectively. The sensitivity was 3 pmol/liter.

**Diagnostic imaging**

MRI of the sellar region was performed using sagittal, coronal, and axial cuts of 2 mm each with gadolinium enhancement. The tumor size
was calculated on the basis of the coronal and sagittal cuts passing through the mean line of both planes; the height of the adenoma was measured from the base. The MRI study was made at time 0 (diagnosis) and at 180 d/6 months (end of treatment) using the same equipment and operator, who was unaware of the specific treatment.

Statistical analysis

All results are expressed as medians and ranges. The comparison between intra- and intergroup ACTH, α-MSH, and RC/C averages were made by means of the nonparametric ANOVA followed by Dunn’s test for multiple comparisons. The intragroup tumor size and pre- and posttreatment results were analyzed with Wilcoxon’s test. The clinical data were evaluated by means of the table of contingency ($\chi^2$ with Yate’s correction) followed by Fisher’s exact test and determination of the odds ratio (OR) between both groups after treatment. Survival curves were constructed and evaluated by means of the log-rank $\chi^2$ test.

Ethical approval

The Ethics Committee of the Faculty of Veterinary Sciences and the Secretary of Sciences of the Universidad de Buenos Aires (UBACYT-V045 project) approved the present study, according to the laws on experimentation in animals in Argentina and World Health Organization recommendations. The signed consent of the dog owners was obtained for participation in the present project.

Results

Levels of plasma ACTH and α-MSH and urinary cortisol in dogs with Cushing’s disease treated with retinoic acid compared with ketoconazole

Before treatment, dogs in both groups had similar levels of ACTH, α-MSH, and urinary cortisol. In the Ktz group, there were no significant changes in ACTH or α-MSH at any of the times studied. However, as shown in Fig. 1A, in the Rx group, a significant decrease was observed in plasma ACTH at 90 d of treatment in comparison with time 0 ($P < 0.01$), and this fall was maintained until the end of the study ($P < 0.01$ for 150 vs. 0 d; $P < 0.001$ for 120 and 180 vs. 0 d). The differences between the treatment groups were statistically significant at 120 d ($P < 0.05$), 150 d ($P < 0.01$), and 180 d ($P < 0.001$). As shown in Fig. 1B, plasma α-MSH also showed a similar reduction with retinoic acid treatment. In the Rx group, a significant reduction was observed at 90 vs. 0 d ($P < 0.05$), remaining significantly suppressed until the end of the study ($P < 0.001$ for 120, 150, and 180 vs. 0 d). Significant differences between the groups were observed at 120 d ($P < 0.05$).

The RC/C ratio (Fig. 1C) decreased significantly in both groups from 120 d; this reduction was more evident in the Rx group and was significantly different between the groups at 180 d ($P < 0.01$). The fall in urinary cortisol in the Rx group paralleled the reduction in plasma ACTH.

Tumor size reduction after retinoic acid treatment

In the Rx group, pituitary adenoma size, as assessed by MRI, was significantly reduced at the end of the treatment compared with the baseline ($P < 0.008$; Fig 2). In only a single case was there no obvious decrease in tumor size. In the Ktz group, no significant variation in the size of the pituitary adenoma was observed (data not shown), correlating with the fact that the pituitary hormones also remained stable.

Fig. 1. Changes in ACTH, α-MSH, and urinary cortisol in dogs with Cushing’s disease treated with 2 mg/kg body weight/d of retinoic acid (isotretinoin 9-cis) (Rx) vs. a control group treated with 20 mg/kg body weight/d of ketoconazole. A, Intra-Rx group vs. time zero: ***, $P < 0.01$ for 90 and 150 d; ***, $P < 0.001$ for 120 and 180 d. There were not significant differences from baseline in the Ktz group. Ktz vs. Rx group: $\phi$, $P < 0.05$ for 120 d Rx vs. 120 d Ktz; $\psi$, $P < 0.01$ for 150 d Rx vs. 150 d Ktz; $\phi\phi$, $P < 0.001$ for 180 d Rx vs. 180 d Ktz. B, Intra-Rx group vs. time zero: $\ast$, $P < 0.05$ for 90 d; ****, $P < 0.001$ for 120, 150, and 180 d. There were not significant differences in the Ktz group. Ktz vs. Rx group: $\phi$, $P < 0.05$ for 120 d Rx vs. 120 d Ktz. C, Intra-Rx group vs. time zero: ***, $P < 0.001$ for 180 d. Intra-Ktz group vs. time zero: ***, $P < 0.01$ for 120 d; **, $P < 0.05$ for 180 d. Ktz vs. Rx group: $\phi\phi$, $P < 0.01$ for 180 d Rx vs. 180 d Ktz. Values are expressed as median and range. ANOVA-Dunn’s test (intra- and intergroup). Open squares (Rx) and triangles (Ktz) represent individual dogs and their variation during treatment.
Survival curve and clinical signs

Of the total number of dogs that were initiated into the study (Table 1), five dogs ceased treatment in the Rx group; two died secondary to complications of Cushing’s disease (tumor apoplexy) and one of cardiac insufficiency, whereas two were removed from the study (one because of footpad hyperkeratosis with pain on weight bearing, the other because of the owner’s decision). By contrast, 11 dogs in the Ktz group died, 10 because of adverse effects related to Cushing’s disease and one case of tumor apoplexy, and one left because of owner’s choice. The time of survival after initiation of treatment was significantly longer in the Rx group compared with the Ktz group, of which more than 50% of the dogs died before 180 d (Fig. 3).

Of the clinical signs monitored, weight was the only parameter studied that did not show significant differences between the groups. During the study period, there was a decline in mean body weight in both groups (−8.98 ± 6.1% in the Rx group vs. −9.2 ± 8.5% in the Ktz group; \( P > 0.05 \), not significant). Retinoic acid induced an improvement in all the clinical signs evaluated (return of estrus, food intake, skin appearance, and hair loss; Table 1). Resumption of normal gonadal activity, observed in 10 of 12 females in the Rx group, occurred between 3 and 5 months after initiation of the therapy, correlating with the observed reduction in the ACTH and urinary cortisol concentrations. At the present moment, some 6–12 months after completion of the trial and cessation of treatment, none of the dogs treated with retinoic acid has shown evidence of recurrence of Cushing’s disease.

Discussion

Ketoconazole is an established treatment for Cushing’s disease, both in the human and in dogs, which operates by interfering with steroid biosynthetic pathways. In this study we show that retinoic acid is at least as effective as ketoconazole, if not more so, but the fall in cortisol excretion is accompanied by a reduction in circulating ACTH and \( \alpha \)-MSH and a reduction in size of the pituitary tumor. This supports the concept that retinoic acid may improve the clinical and biochemical signs of Cushing’s disease by a direct action on the tumorotic corticotroph, as suggested by earlier studies in rodents (25). In dogs, two convertase enzymes are involved in processing the POMC gene products; one of these systems is active in the cells of the anterior pituitary that secrete ACTH, whereas the other is located in the intermediate-lobe cells that predominantly secrete \( \alpha \)-MSH (34). In the present study, a reduction in both hormones was seen, suggesting that retinoic acid acts on the pituitary adenoma arising not only from the anterior lobe but also from the normal intermediate lobe. However, it cannot be excluded that the \( \alpha \)-MSH originated form the tumor per se. This modulation of the activity of Cushing’s disease was associated with increased survival, with only a single animal dying from cardiac failure, a complication possibly related to hypercortisolemia. By contrast, in the Ktz group, more than 50% of the animals died before completing the treatment, usually from complications of the glucocorticoid excess, with survival similar to that previously documented for ketoconazole (28). We would attribute this to more effective control of the hypercortisolemia in the Rx group.

There was a remarkable improvement in clinical signs in the Rx group. Fluid intake fell, presumably as a consequence of an increase in renal blood flow and inhibition of the salt-retaining effects of cortisol (27), and there was a return of normal gonadal function with disease control (35). It would therefore appear that retinoic acid treatment, via a reduction in ACTH and cortisol levels, leads to normalization of the gonadal axis and the estrous cycle. Reversal of anestrus occurred at 90 and 150 d after the initiation of the therapy, coincident with a reduction in ACTH and RC/C. This may
TABLE 1. Changes in clinical signs observed in dogs with Cushing’s disease treated with 2 mg/kg body weight/d of retinoic acid compared with 20 mg/kg weight/d ketoconazole

<table>
<thead>
<tr>
<th>Clinical signs</th>
<th>Retinoic acid</th>
<th>Ketoconazole</th>
<th>OR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Survivor at end of treatment (death or abandon/alive)*</td>
<td>Pretreatment 5/17</td>
<td>Posttreatment 12/8</td>
<td>0.2</td>
</tr>
<tr>
<td>Water intake (abnormal/normal intake)*</td>
<td>Pretreatment 20/2</td>
<td>Posttreatment 0/17</td>
<td>0.018</td>
</tr>
<tr>
<td>Estrus state (anestrus/estrus)*</td>
<td>Pretreatment 12/0</td>
<td>Posttreatment 2/10</td>
<td>0.05</td>
</tr>
<tr>
<td>Polyphagia (with/without)*</td>
<td>Pretreatment 18/4</td>
<td>Posttreatment 2/15</td>
<td>0.133</td>
</tr>
<tr>
<td>Skin (abnormal/normal)*</td>
<td>Pretreatment 16/6</td>
<td>Posttreatment 5/12</td>
<td>0.14</td>
</tr>
<tr>
<td>Hair (abnormal/normal)*</td>
<td>Pretreatment 13/9</td>
<td>Posttreatment 3/14</td>
<td>0.21</td>
</tr>
<tr>
<td>Abdomen (prominent/normal)*</td>
<td>Pretreatment 21/1</td>
<td>Posttreatment 11/6</td>
<td>1.1</td>
</tr>
<tr>
<td>Weight (% reduction from pretherapy)*</td>
<td>Pretreatment –8.98 ± 6.1</td>
<td>Posttreatment –9.2 ± 8.5</td>
<td>0.133</td>
</tr>
</tbody>
</table>

Pretreatment Rx group n = 22; posttreatment Rx group n = 17; pretreatment Ktz group n = 20; posttreatment Ktz group n = 8. All data are expressed as number of cases (with alteration in sign/improved or normal sign). Contingency table and Fisher’s exact test were used for all statistical analyses. OR represents odds ratio for posttreatment Rx vs. Ktz group.

* P < 0.03 post-Rx vs. post-Ktz. In the Rx group, two dogs died from illness, one of them at the end of treatment (both had macroadenomas), two dogs abandoned the study, and one dog died from a cardiac failure previously diagnosed. All deaths of dogs in the Ktz group were secondary to Cushing’s disease.

** P < 0.001 post-Rx vs. pre-Rx and post-Rx vs. post-Ktz.

* P < 0.03 post- vs. pretreatment Rx. From 12 unneutered bitches in both groups, 10 of 12 in the Rx group and one of four in the Ktz group (seven bitches died during the treatment time) normalized its reproductive function. Sexual function returned 3–4 months from the beginning of therapy with retinoic acid and 5 months with ketoconazole in the single bitch.

* P < 0.0001 post- vs. pretreatment Rx. Pregnant abdomen is caused by a large amount of visceral fat and enlargement of the liver. Reduction of the abdominal fat results in decreased size.

A reduction was observed in both Rx and Ktz groups with respect to the initial values, with no significant difference between groups.

be contrasted with the results observed in the Ktz group, in which estrous cyclicity was restored in only a single female. In humans, too, Cushing’s syndrome is associated with gonadal dysfunction, which improves with effective therapy (36).

By contrast, a similar weight reduction was observed in both groups, presumably secondary to the fall in cortisol with both drugs. An interaction between retinoic acid (through the RXR) with PPAR-γ has also recently been described (37). This interaction regulates the process of gluconeogenesis in adipose tissue by the expression of the cytosolic form of phosphoenolpyruvate carboxykinase (38). Thus, the Rx group would have an additional effect because of the interaction of retinoic acid and PPAR-γ in controlling intra-adipocyte gluconeogenesis.

No adverse events with retinoic acid were recorded, except for one case of footpad hyperkeratosis, and there was no evidence of hepatotoxicity during or after the study in terms of hepatic enzyme abnormalities. Based on these data, the dose of retinoic acid used (2 mg/kg body weight/d) was not only effective but also appeared to be safe.

The mode of action of retinoic acid involves an interaction with retinoic acid receptor and RXR (18, 29). Earlier work has demonstrated that retinoic acid blocks the activation of POMC transcription by the orphan receptors Nur77 and Nur11, that this process is particularly potent in tumors as opposed to normal tissue, and that it is associated with a decrease in adenosomatous proliferation and increased apoptosis (25). The dose requirements in rodents is within an order of magnitude of that used in the current studies and not far removed from therapeutic doses of this drug used in the human for other conditions (17, 25). The current findings suggest that studies in rodents can be extrapolated to the dog and are likely to be therapeutically relevant to the human.

In summary, we describe the effect of retinoic acid in
Cushing’s disease in dogs, where a well tolerated dose regimen was highly effective in reducing the overactivity of the pituitary-adrenal axis and was associated with shrinkage of the corticotrophinoma. Retinoic acid produced an improvement in all clinical signs monitored and increased survival compared with ketoconazole. This study highlights the possibility of using retinoic acid as a medical therapy for ACTH-secreting tumors in human patients, for whom there is no generally effective pharmacological therapy. Long-term clinical trials in patients will be needed to determine whether retinoic acid has the same observed effects that we have shown in this animal model.

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