Endocrine and metabolic characteristics of polyoma large T transgenic mice that develop ACTH-producing pituitary tumors

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Some benign tumors result in clinical consequences similar to those of malignant neoplasms. Such tumors may cause symptoms due to growth within a closed compartment such as the skull, obstruction of ducts and vessels, or they may produce large amounts of biologically active peptides.

Pituitary tumors arise from the various cell types in the pituitary gland and are almost always morphologically benign. Their growth may affect the function of neighboring structures and/or result in excessive production of anterior pituitary hormones. Patients with Cushing’s disease have pituitary tumors that secrete pathological amounts of adrenocorticotropic hormone (ACTH), with permanent overstimulation of the adrenal cortex. The resulting elevated cortisol levels have consequences for fat, protein, and carbohydrate homeostasis, often leading to hypertension, obesity, and hyperglycemia.

Recently, we developed an animal model for Cushing’s disease. Transgenic mice with the polyoma large T (PyLT) antigen complementary deoxyribonucleic acid (cDNA) develop pituitary adenomas that are fatal at approximately 1 year of age. These adenomas express the transgene at the messenger ribonucleic acid level (unpublished data), and we have shown that they are ACTH-producing. Here we report that these transgenic mice, as well as immunocompetent histocompatible mice carrying subcutaneous transplants of the pituitary tumors have pathologically increased levels of ACTH in plasma, and we describe some accompanying biological effects of body weight, blood glucose, and water balance. Both the transgenic mice and those with tumor transplants display histopathological and endocrinological features that make them relevant models for studying Cushing’s disease in humans.

Materials and Methods

Transgenic Mice

Transgenic mice were generated by microinjection of the polyoma early region promoter linked to the cDNA-encoding PyLT into the male pronuclei of fertilized eggs. Transgenic pups in a litter were identified by the presence of PyLT in genomic DNA isolated from tail biopsies taken at 2 weeks of age. Nontransgenic siblings were used as control animals in this study. The mice were kept in a
facility with humidified and filtered air together with other transgenic lineages approved by the American Association for Accreditation for Laboratory Animal Care. They had access to food and water ad libitum, and were inspected daily. The mice were anesthetized with an intraperitoneal injection of 0.65 ml 2.5% avertin per 30 g animal weight.*

Transplant Mice

Pituitary tumors from the transgenic animals (PyLT-1) were minced with sterile scalpel blades and forced through a 19-gauge hypodermic needle. Then, 0.1 ml tumor slurry was injected subcutaneously into young B6D2F1 nontransgenic female mice† and passed four times. In addition, another fourth passage was set up using tissue cubes approximately 2 mm in size instead of the tumor slurry.

In each experiment four to 15 test animals received transplants and three to five mice that did not receive tumor were used as controls. All mice receiving transplants were females, except in the third passage, in which both genders were used. The mice receiving transplanted tumor tissue were weighed every 2 weeks, starting on the day of the transplantation. The implant site was inspected regularly and from the time a tumor was palpable, the experimental and the control mice were anesthetized every 2nd week and two perpendicular tumor diameters were measured with calipers. Tumor volumes were calculated according to the formula \(0.5 \times \text{length} \times \text{width}^2\), and growth curves were constructed.‡

Blood Sampling

Blood samples for a time-course study were obtained by sacrificing transgenic mice at ages 4 and 9 months together with their age- and sex-matched controls. In addition, blood was withdrawn from all mice that became clinically ill during the experiment. Each age group contained four to five experimental and three to six control animals of each gender. The mice that received transplanted tumor were sacrificed at ages 6 months (third passage, six females), 9 months (third passage, two males and three females), and approximately 12 months (second passage, five females). All blood samples were withdrawn between noon and 4 p.m. Two 9-month-old mice with third passage transplants were sacrificed at 2 and 7 weeks after their tumors were removed for establishing the fourth passage series.

Animals were brought from the animal room to a separate autopsy room 2 hours or more prior to autopsy. Autopsies were started and completed between noon and 4 p.m. A heart puncture was performed in anesthetized mice with a 1-ml syringe and 22-gauge hypodermic needle premixed with 1000 U/ml heparin. After sampling, the mice died of hypovolemia. The blood was transferred to chilled microfuge tubes and immediately centrifuged at 8000 rpm for 10 minutes. The plasma was transferred to clean, chilled microfuge tubes and stored at \(-70^\circ\text{C}\).

Analysis of ACTH

Levels of ACTH in plasma were measured using a double-antibody radioimmunoassay. For these analyses, human ACTH antisera (AFP 6328031), human ACTH for iodination (AFP 2938C), and rat reference preparation (rACTH-RP-1) were used.

Blood Glucose Levels

Blood glucose was measured with Glucofilm test strips and a glucometer.‡ Mice were held by the tail in a restrainer, and 1 to 2 mm of the distal part of the tail was excised. One drop of blood was placed on the strip, and the glucose level was measured according to the manufacturer’s instructions. Normal control samples were used to test the system for each new batch of test strips. The mice were denied food and water the night before the sampling. In one study, fasting blood glucose levels were measured at 8 a.m. in three female and three male PyLT-1 transgenic mice regularly for 3 months from the age of 9 months. In another experiment, a glucose tolerance test was performed on mice with 7-month-old third passage pituitary tumors. Nine tumor-bearing and six control mice of each sex were denied food and water overnight, and at 8 a.m. the blood glucose level was measured. Thereafter, 1 mg glucose per gram body weight was injected intraperitoneally, and blood glucose levels were measured hourly for 4 hours.

Urine Output

Groups of two to four mice were kept in metabolic cages for mice§ for 3 to 4 days. The inner funnel of the cage was coated with Prosil-28‖ according to the manufacturer’s instructions. Water intake in milliliters and urinary output in grams were measured every 24 hours.

Hormone Receptor Analyses

Tumor material from 9-month-old mice transplanted 8 months earlier with third passage PyLT-1 pituitary tumors was used. The mice, three males and three females, had 10- to 22-mm large subcutaneous transplant tumors. One primary pituitary tumor from a sick PyLT-1 female was also investigated. The tumor material or pituitary glands were immediately put into microfuge tubes, frozen in liquid nitrogen, and stored at \(-20^\circ\text{C}\). Cryostat sections were cut and used for estrogen and progesterone receptor staining, using kits and recommendations from the manufacturer.* Positive and negative control samples were included.

Statistical Analysis

The relationship between the quantitative findings in the experimental and the control animal groups was evaluated nonparametrically using the Mann–Whitney two-sample test in a graphics program.† A value of \(p < 0.05\) was considered significant.

Results

Clinical Symptoms and Pathology

To study the time course of the morphological and the endocrinological changes, symptom-free transgenic mice at 4 and 9 months of age were studied in addition to symptomatic transgenic mice and compared to matched, nontransgenic controls. When the mice became clinically ill they developed a spinal hump in the thoracic region and an unsteady gait, and they had difficulty in reaching food and water. Sick mice deteriorated rapidly and were killed. The mean age at the time of autopsy for these animals was 15.5 months for males and 13.1 months for females.‡

All clinically ill PyLT-1 transgenic mice had large pituitary tumors (approximately 5 mm in greatest diameter) that compressed neighboring structures (Fig. 1A). Microscopically the tumors were characterized by nests and cords of tumor cells with minimal atypia and few mitotic figures (Fig. 1B). The tumors were surrounded by a

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* Avertin anesthetic is a weight/volume formulation composed of 5 g 2,2,2 trichloroethanol from Aldrich Chemical Company Inc., Milwaukee, Wisconsin in 10 ml 2-methyl-2-butanol obtained from Fluka Chemical Corp., Ronkonkoma, New York.

† B6D2F1 nontransgenic female mice were supplied by Jackson Laboratories, Bar Harbor, Maine.

‡ Ames Glucofilm and Model 3 Glucometer glucose testing equipment were manufactured by Miles Inc., Elkhart, Indiana.


‖ Prosil-28 supplied by PGC Scientific, Gaithersburg, Maryland.

* Estrogen and progesterone receptor staining kits supplied by Abbott Laboratories, North Chicago, Illinois.

† NCSS 5.1 Graphics program provided by Dr. J. L. Hintze, Kaysville, Utah.

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Pseudocapsule, and invasive growth into the brain parenchyma was not detected. The mice that received transplants had subcutaneous tumors of a similar histopathology but mitoses were easily identified and greater in number. In mice with large transplanted tumors, focal areas of intratumoral necrosis were apparent. The tumor cells were tested for immunoreactivity against a battery of anterior pituitary peptides as reported previously.4, 5 Many

Fig. 1. Macroscopic and microscopic tumor pathology.  A: Photograph showing representative macroscopic appearance of a large pituitary adenoma in a polyoma large T-1 (PyLT-1) transgenic mouse.  B: Photomicrograph presenting an overview of the light microscopic morphology of the PyLT-1 pituitary tumor, with neoplastic cells arranged in cords and nests, H & E, ×100.  C: Photomicrograph of a PyLT-1 pituitary tumor showing minimal cellular atypia and an adenomatous organization, H & E, ×400.  D: Photomicrograph demonstrating the ACTH-positive immunoreactivity of the tumor in a characteristic field. AEC (3-amino-9-ethylcarbazole) and hematoxylin, ×400.
tumor cells showed a weak and rarely an intense positive immunoreactivity for ACTH in the cytoplasm (Fig. 1C and D), whereas no reactivity was evident in the cells of interest with antibodies against growth hormone, prolactin, luteinizing hormone, follicle-stimulating hormone, or thyroid-stimulating hormone. The immunocytochemical findings were similar in the primary and the transplanted tumors.

**Plasma ACTH Levels**

Table 1 gives the plasma ACTH concentrations in control, transgenic, and transplanted mice according to age and sex. The median ACTH values for 12-month-old female transgenic, transplant, and control mice have been published previously. The baseline value was less than 10 μg/L ACTH in plasma of the control mice of both sexes and in all age groups studied. In 4-month-old transgenic mice none of the samples showed increased ACTH levels. At 9 months, a moderate increase was seen in three of four samples from males, and in one of the four samples from females a markedly increased hormone level was found (170 μg/L). The latter sample was from the only 9-month-old mouse with a microadenoma. Both male and female clinically ill (≥ 12 months) transgenic mice had significantly increased ACTH levels relative to the control animals. Furthermore, the clinically ill female PyLT-1 mice had significantly higher plasma ACTH concentrations than the corresponding male mice (p = 0.0275).

All but one of the 6-month-old mice with transplanted tumors had markedly increased ACTH levels in plasma, in contrast to the situation in 4-month-old transgenic mice. The one 6-month-old animal with a normal value had no palpable tumor at the time of autopsy, but a small tumor (1–2 mm) was found by dissection and confirmed histologically to be adenomatous tissue. Tumors from two female tumor-bearing mice were used for establishing a fourth passage series of tumor transplants. These donor mice were subjected to autopsy and blood sampling 2 and 7 weeks after their tumors were removed for passaging. The ACTH findings in these two mice were 109 μg/L (2 weeks) and 21 μg/L (7 weeks).

The ACTH data in Table 1 show that PyLT-1 transgenic mice with micro- or macroadenomas of the pituitary had significantly increased levels of ACTH in the blood. The immunocompetent mice with subcutaneous tumor transplants had even higher ACTH levels.

**Blood Glucose Levels**

Patients with Cushing’s disease may develop hyperglycemia and diabetes. We therefore studied the fasting blood glucose levels in PyLT-1 transgenic mice from the age of 8 through 12 months to analyze possible blood glucose responses to the pathological ACTH output from the tumor. The data in Table 2 show that there was no significant increase in glucose levels in the age period covering neoplastic transformation in the pituitary gland. This indicates that the increased ACTH secretion in PyLT-1 transgenic mice had no significant impact on the fasting blood glucose levels.

In the glucose tolerance test, mice with transplanted tumors were compared to B6D2F1 control mice. The control mice show a physiological response to the glucose challenge with return to the fasting level after approximately 2 hours (Table 3). The transplant mice show a similar response but the mean glucose level is higher at each time point. The difference is significant at each point in spite of the wide range of the results in the tumor group. This finding indicates that the transplant mice have a stressed carbohydrate metabolism with hyperglycemia, but overt diabetes mellitus with a delayed return to fasting level after glucose challenge has not occurred.

**Urine Output**

The adrenal production of glucocorticoids, and to a lesser extent the mineralocorticoids, are regulated by ACTH. It was therefore of interest to examine whether increased ACTH levels had consequences for urinary out-
Pituitary tumors in transgenic mice

| TABLE 4 |
|------------------|------------------|------------------|
| **Urine output and drinking volume per 24 hours in female PyLT-1 transgenic mice, transplant mice, and control mice** |
| Mice      | Age (mos) | Mean Urinary Output | Mean Drunking Vol. ml |
| control   | 6–7      | 6.3 (1.1)            | 15.4 (3.4)            |
| PyLT-1    | 6        | 7.2 (0.7)            | 15.0 (3.7)            |
| PyLT-1    | 10       | 2.2 (1.1)            | 4.5 (1.2)             |

* Numbers in parentheses are standard deviations.
† Urinary output is measured in g/100 g body weight.

Put and water intake. At 6 months of age, prior to symptoms and signs of neoplastic transformation in the pituitary, no difference was found between PyLT-1 and control animals (Table 4). In 10-month-old transgenic mice, however, the urine output and water intake were significantly reduced compared to both control mice and younger PyLT-1 animals.

**Tumor Growth and Body Weight Changes**

To quantitate the growth rate of the transplant tumors, mice with fourth passage tumors (cubes) were studied. The mean tumor volume is presented in Fig. 2. By 82 days after inoculation the volume was 38 mm³ and increased slightly until approximately 105 days. Thereafter, a steady increase in the tumor volume was observed with a calculated doubling time of approximately 6 days. The sigmoid shape of the growth curve resembles closely the Gompertzian growth pattern typically seen in solid tumors.11

Patients with Cushing’s disease usually gain weight. We studied the body weight of mice with fourth passage transplant tumors. No weight difference was found between mice with transplanted tumors and age-matched control animals until approximately 95 days after tumor inoculation (Fig. 2). From this point, however, the weight of the transplant mice increased significantly relative to that of the control animals. Both male and female mice developed a neck hump of increased fat deposition, and this sign was more apparent in the female mice. Together, the data show that when the subcutaneous tumors were established, with a mean tumor volume of approximately 50 mm³, the transplant mice responded with a pathological weight increase.

**Sex Hormone Receptors**

Because of the observed differences in the age of onset of symptoms and tumor size between male and female PyLT-1 transgenic mice, we examined tumor cells for the presence of estrogen or progesterone receptors. Immunocytochemical studies did not detect such receptors in primary pituitary tumors or in transplanted tumors (data not shown). The indications of biological differences in tumor development between the sexes, therefore, may not be related to such sex hormone receptors.

**Discussion**

The present study demonstrates that PyLT-1 transgenic mice as well as mice receiving tumor transplants have some of the metabolic and endocrine disturbances characteristic of Cushing’s disease. All tumor-bearing mice showed pathologically increased plasma ACTH levels clearly associated with the growth of the PyLT-1 pituitary tumors in the animals. A small increase in fasting blood glucose levels was found in mice with transplanted tumors. Mice with transplanted tumors increased their body weight beyond the corresponding control mice from the time the subcutaneous tumors were palpable. These findings are indicative of biological responses to increased ACTH in transgenic and transplant mice.

Several transgenic lineages of animals with various promoter/enhancer regions and oncogenes develop pituitary tumors. It is unclear whether the pituitary gland is a preferred site for neoplasia in transgenic systems. Murphy, et al.,9 have reported a model that mimics human multiple endocrine neoplasia by using the upstream sequences of the bovine vasopressin gene linked to simian virus-40 T antigen. Their mice developed pancreatic B-cell tumors and pituitary tumors with pleomorphic nuclei and frequent mitotic figures. Windle, et al.,13 used the same oncogene under the regulator control of the human glycoprotein hormone α subunit. Their mice developed anterior pituitary tumors, and a clonal cell line from the tumor secreted α subunit protein. Thus, this system resembles the class of human anterior pituitary tumors termed nonfunctioning adenomas. In a third model, pituitary hyperplasia with ele-
vated serum growth hormone levels and clinical gigantism was demonstrated in transgenic mice with a rat growth hormone promoter–cholera toxin construct. Thus, transgenic mouse models are available for the study of various human diseases involving the anterior pituitary: multiple endocrine neoplasia, nonfunctional pituitary adenomas, gigantism, and Cushing’s disease.

To measure ACTH in mouse plasma we used an assay for rodent ACTH employing a rat reference preparation. The assay used for human samples was unsuitable because of the low affinity of the human antibody for mouse ACTH, as detected in a pilot study (data not shown). Sick PyLT-1 mice were found to have pathologically raised plasma ACTH levels, median 180 μg/L in males and 685 μg/L in females. Another transgenic lineage with the corticotropic-releasing factor (CRF) gene under the control of the metallothionein promoter has been reported by Stenzel-Poore, et al. These transgenic mice also displayed physical and endocrinological changes similar to those of patients with Cushing’s disease.

Transgene expression in the cells expressing the endogenous CRF gene and plasma levels of ACTH were elevated fivefold compared to the control animals in the study by Stenzel-Poore, et al. Whereas the CRF transgenic mice had an ACTH mean at 100 pg/ml, our assay gave a data set in a completely different concentration range. Although the ACTH results for CRF transgenics resemble the human data for pituitary adenomas, the range seen for the PyLT-1 transgenics and the transplants can be found in human patients with ectopic ACTH secretion. One important factor to be considered is the inherent stress at sampling. Our blood sampling procedure exposed the animals to higher stress levels. Thus, our data represent stress responses in the various groups and ages. Even so, the data correlate with the histopathological findings in that mice with no pathological changes in their pituitaries had low plasma ACTH levels, and PyLT-1 mice 9 months and older with micro- and macroadenomas showed dramatic increases in plasma ACTH.

The data indicate a possible sex difference in the PyLT-1 transgenic mice. Clinically ill female mice have significantly higher ACTH values than the males, the mean life span was somewhat shorter in female PyLT-1 compared to males, and the subcutaneous tumor transplants reached a larger maximum tumor diameter in female recipients. We therefore raised the question of whether differential expression of estrogen or progesterone receptors could be involved in the sex differences. However, we did not detect these receptors in the pituitary or transplant tumors, contrary to a finding in a previous report on human pituitary tumors.

The PyLT-1 pituitary tumors have a latency of approximately 1 year (unpublished data), but from the time the transgenic mice started to show clinical symptoms of the pituitary adenoma, they deteriorated rapidly. Also, when the tumors were transplanted subcutaneously to immunocompetent mice, a long latency period before tumor growth was observed. Growth was rapid, however, from the time a small tumor was palpable. Thus, the tumor growth data are in accordance with our clinical experience and previous pathology data. The weight data showed that mice in the transplanted tumor group with a palpable and growing subcutaneous tumor had a weight increase beyond that of matched control animals. This parameter may therefore be used as a crude screening test for the hypercorticotropin state in the transplant model.

The analysis of urine output and water intake showed that 6-month-old PyLT-1 mice did not differ from nontransgenic control mice. At 10 months of age, however, the water intake and output was significantly reduced. Based on our previous timed study of tumor histology, the mice have microadenomas at this age. The explanation for the reduced water intake is not known and various mechanisms are possible. Among these are mobility and voluntary muscle control, as the mice may have difficulty reaching the water nipple, hypothalamic disorders, and, at present, unknown perturbations of the PyLT-1 transgenic lineage.

We have previously shown that the PyLT transgenic mice develop ACTH-immunoreactive pituitary tumors with important characteristics in common with Cushing’s disease in humans. The PyLT-1 transgenic mice and the transplant mice showed morphological differences in the adrenal glands, with the findings in the transplant mice similar to those in human Cushing’s disease. The present work documents pathologically increased plasma ACTH levels in both primary transgenic and transplant mice. The transplant mice had higher plasma ACTH levels than the transgenic mice. Furthermore, only the transplant mice showed changes in carbohydrate and fat metabolism with an elevated fasting blood glucose level and a pathological weight gain. Thus, the mice with subcutaneous transplants of the PyLT-1 pituitary tumor have many features in common with Cushing’s disease including elevated plasma ACTH, secondary adrenal cortex changes, blood glucose changes, and obesity. This model may therefore be useful for the study of pituitary/adrenal dysfunctions and therapeutic regimens.

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