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Dopamine Receptor Expression and Function in Corticotroph Pituitary Tumors

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The role of dopamine agonist treatment in corticotroph pituitary tumors is controversial. The aim of this study was to evaluate D_2 receptor expression in 20 corticotroph pituitary tumors and to correlate it to the *in vitro* effect of dopamine agonists on ACTH secretion and the *in vivo* effect of short-term cabergoline treatment on cortisol secretion. D_2 expression was evaluated by receptor-ligand binding, immunohistochemistry, and RT-PCR. A 50% or more decrease in daily urinary cortisol levels was considered a significant clinical response. At receptor-ligand binding, specific binding of [^{125}I]epidepride was found in 80% of cases. At immunohistochemistry, specific D_2 immunostaining was found in 75% of cases. D_2 expression was found in 83.3% of cases (D_{2long} in 40%, D_{2short} in 20%, and both in 40%) by RT-PCR. Significant *in vitro* inhibition of ACTH secretion was found in 100% of D_2-positive cases, but not in 100% of D_2-negative cases by either bromocriptine or cabergoline. A significant *in vivo* inhibition of cortisol secretion after 3-month cabergoline treatment was found in 60%, although a normalization of cortisol secretion was found in 40% of cases. All cabergoline-responsive cases were associated with D_2 expression, whereas all noncabergoline-responsive cases but one were not associated with D_2 expression. In conclusion, functional D_2 receptors were expressed in approximately 80% of corticotroph pituitary tumors. The effectiveness of cabergoline in normalizing cortisol secretion in 40% of cases supports its therapeutic use in the management of Cushing's disease. (*J Clin Endocrinol Metab* 89: 2452–2462, 2004)

DOPAMINE IS THE predominant catecholamine neurotransmitter in the human central nervous system where it controls a variety of functions, including cognition, emotion, locomotor activity, and regulation of the endocrine system (1). Dopamine also plays multiple roles in the periphery as a modulator of cardiovascular and renal function, gastrointestinal motility, and hormone synthesis and secretion (1). The various actions of dopamine are mediated by five specific receptors (D_1–D_5), which can be subdivided into two different receptor families on the basis of their biochemical and pharmacological characteristics: D_1-like, including D_1 and D_5, and D_2-like, including D_2, D_3, and D_4 receptors (1). The different dopamine receptor (DR) subtypes have different distributions and play different roles in the various organs and tissues (1).

D_2 receptor is expressed in the anterior and intermediate lobes of the pituitary gland (2, 3), where it mediates the tonic inhibitory control of hypothalamic dopamine on prolactin (PRL) and MSH secretion, respectively (4–6). The presence of a functional D_2 receptor on tumor PRL-secreting cells (7–10) led to a major therapeutic application in the treatment of PRL-secreting pituitary tumors. Indeed, medical therapy with dopamine agonists represents the first choice treatment of this type of pituitary tumors, being effective in suppressing PRL secretion and inducing tumor shrinkage (11–13). Moreover, among the different dopamine agonists, the more recently developed drug, cabergoline, has been demonstrated to be more effective than the most widely used drug, bromocriptine, in the treatment of PRL-secreting pituitary tumors (14, 15).

Treatment with bromocriptine has been also investigated in ACTH-secreting or corticotroph pituitary tumors, although with controversial results (12, 16). However, no study has ever evaluated DR expression and the effect of cabergoline treatment in controlling the ACTH and cortisol hypersecretion associated with corticotroph pituitary tumors.

The current study was designed with a 2-fold purpose: 1) to evaluate DR expression in corticotroph pituitary tumors by different techniques, namely receptor-ligand binding (R-LB), immunohistochemistry (IHC), and RT-PCR; and 2) to correlate DR expression to the *in vitro* effect of dopamine agonists on ACTH secretion in cultured tumor corticotroph pituitary cells and to the *in vivo* effect of cabergoline treatment on ACTH and cortisol secretion in patients with Cushing's disease (CD), characterized by excessive endogenous ACTH and cortisol secretion induced by a corticotroph pituitary tumor (17).

Abbreviations: CD, Cushing's disease; DR, dopamine receptor; HPRT, hypoxanthine ribosyl transferase; IHC, immunohistochemistry; LiDS, lithium dodecyl sulfate; NS, nonspecific; oligo(dT)$_{25}$, oligo(deoxythymidine)$_{25}$; poly(A)$^+$, polyadenylated; PRL, prolactin; R-LB, receptor-ligand binding; T, total; TBS-T, Tris-buffered saline-Tween; WGA, wheat-germ agglutinin.

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Subjects and Methods

Patients

Twenty patients (16 women and 4 men, 25–60 yr of age) with a diagnosis of CD entered the study after their informed consent had been obtained. Ten patients were admitted to Department of Internal Medicine, Erasmus Medical Center (Rotterdam, The Netherlands; group 1), and 10 were admitted to Department of Molecular and Clinical Endocrinology and Oncology, Federico II University (Naples, Italy; group 2) over a period of 2 yr. The patients of group 1 were retrospectively selected on the basis of the availability of a tumor specimen collected and frozen at the time of neurosurgery; the patients of group 2 were selected on the basis of the presence of persistent or recurrent disease after neurosurgery for inclusion in a prospective study. The diagnosis of CD was based on 1) increase in daily urinary cortisol excretion with inappropriately high plasma ACTH concentrations; 2) increase in basal serum cortisol concentrations with lack of the physiological circadian rhythm; 3) failure of urinary and serum cortisol suppression after low dose, but greater than 50% decrease after high dose oral dexamethasone suppression test or a decrease in serum cortisol of at least 7 μg/dl in the 7-h continuous iv dexamethasone suppression test (18–20). The diagnosis of CD was supported by the evidence of a pituitary tumor at magnetic resonance imaging of pituitary gland or at bilateral inferior petrosal sinus sampling (20, 21). Indeed, five of the 20 patients had pituitary macroadenoma, 10 had microadenoma, and the remaining five patients had normal pituitaries; the pituitary source of ACTH hypersecretion in these five patients was confirmed by bilateral inferior petrosal sinus sampling. All patients were subjected to neurosurgical operation by the transsphenoidal approach for the removal of the pituitary tumor. The histological and immunohistochemical study of the tumor removed by neurosurgery documented a corticotroph pituitary lesion (adenoma or hyperplasia) in all cases, definitely confirming the diagnosis of CD in the totality of patients. After neurosurgery, clinical, hormonal, and radiological remission of CD was documented in 12 patients, whereas disease persisted in the remaining eight patients. Moreover, disease recurrence occurred in six of the 12 remitted patients in the study 1–3 yr after neurosurgery. The patients' profiles are shown in Table 1.

Samples

Pituitary tumor specimens were obtained at the time of tumor excision by neurosurgery. Samples of these specimens were taken fresh directly at the operation. They were fixed in 10% paraformaldehyde overnight and embedded in paraffin for the IHC study and/or quickly frozen on dry ice and stored in a freezer at −80 C for R-LB and/or RT-PCR studies. In selected cases, a sample was also used for the establishment of a pituitary tumor primary culture. The study included only samples in which the tumor tissue represented at least 90% of the section at the histological evaluation to exclude the influence of normal pituitary tissue on the results of the studies.

Study design

The study protocol was diversified in the two different groups of patients. In group 1, D_2 receptor expression was evaluated by R-LB and IHC studies. DR subtype expression was evaluated by RT-PCR in five of 10 cases. In group 2, D_2 receptor expression evaluated by IHC was correlated to the *in vitro* effect of dopamine agonists (bromocriptine and cabergoline) on ACTH secretion and to the *in vivo* effect of 3-month cabergoline treatment on cortisol secretion. DR subtype expression was evaluated by RT-PCR in seven of 10 cases and also correlated to the results of the *in vitro* and *in vivo* functional studies. The protocol was in accordance with the Helsinki Doctrine on Human Experimentation, and it was approved by the local Ethics Committees.

R-LB

The R-LB study was performed on tissue samples as previously reported (22). The frozen tissue samples were cut in 10-μm-thick sections. These sections were mounted onto precleaned, gelatin-coated microscope glass slides and stored in a freezer at −80 C for at least 3 d before the experiment to improve adhesion of the tissue to the slide. The D_2 analog [^{125}I]epidepride (Radiopharmaka, Seibersdorf, Austria) was used as radioligand. The sections were preincubated at room temperature for 10 min in binding buffer [50 mM Tris-HCl (pH 7.7), 120 mM NaCl, 5 mm KCl, 2 mM $CaCl_2$ $2H_2O$, 1 mM $MgCl_2$ $6H_2O$, and 0.1% ascorbic acid]. Thereafter, they were incubated for 60 min at room temperature in binding buffer with [^{125}I]epidepride. Nonspecific binding was determined in a sequential section in the presence of excess unlabeled cabergoline (1 μM). A sample was considered positive for [^{125}I]epidepride binding when the signal was displaced by more than 50%. The incubated sections were washed twice for 5 min each time in binding buffer. After a short wash in distilled water to remove salts, the sections were air-dried and exposed to Kodak Biomax film (Eastman Kodak, Rochester, NY) or HyperFilm-3H (Amersham Pharmacia Biotech, Houten, The Netherlands) for 7–15 and 30–60 d, respectively, in x-ray cassettes. Histological evaluation was performed on hematoxylin-eosin-stained sequential cryostat sections. In addition, a positive control, represented by a sample of rat brain cut at the level of the basal ganglia, and negative controls, represented by rat tissues known to have no expression of DRs, were used in each experiment. The binding signals obtained were analyzed densitometrically using a computer-assisted image-processing system and were quantified by calculating the ratios between the regions of interest delineated on the total (T) and nonspecific (NS) binding sections. Using the T/NS ratios, the amount of binding in every section was graded as negative (−), for T/NS ranging from 0–1.9, moderately positive (+) for T/NS ranging from 2–3, and strongly positive (2+) for T/NS greater than 3.

IHC

The IHC study was performed on tissue samples according to a previous report (23). The formalin-fixed and paraffin-embedded tissue samples were cut into 5-μm-thick sections. These sections were deparaffinized, dehydrated, exposed to microwave heating in citric acid buffer at 100 C for 15 min, rinsed in tap water, followed by PBS, and subsequently incubated for 15 min in normal goat serum (1:10 dilution in PBS and 5% BSA). The sections were then incubated overnight at 4 C with rabbit antihuman D_2 receptor polyclonal antibody (Chemicon International, Temecula, CA) in a dilution of 1:500. A standard streptavidin-biotinylated alkaline phosphatase or -peroxidase complex (ABC kit, Biogenix, San Ramon, CA) was used to visualize the bound antibodies. Negative controls for the IHC included 1) omission of the primary antibody; and 2) preabsorption of the antibodies with the respective immunizing receptor peptides (at a concentration of 100 nm), both performed in sequential sections. Immunostaining for ACTH and PRL as well as GH, TSH, FSH, and LH was also performed on sequential sections using specific antibodies at the standard dilution. Histological evaluation was performed on hematoxylin-eosin-stained sequential sections. Positive and negative controls were represented by D_2 receptor immunostaining on sections of dopamine agonist-sensitive and -resistant, PRL-secreting pituitary tumors, respectively and were carried out in the same experiments of the corticotroph pituitary tumors. The specificity of the D_2 receptor antibody was tested by immunoblotting using a corticotroph pituitary tumor sample.

Immunoblotting

The immunoblotting was performed on tissue samples according to a previous report (24). Membranes were extracted from a frozen tissue sample. The tissue sample was suspended in an ice-cold Tris-buffer [10 mM Tris-HCl (pH 7.6), 5 mM EDTA, 3 mM EGTA, 250 mM sucrose, 1 mM phenylmethylsulfonylfluoride, 10 μg/ml leupeptin, 10 μg/ml soybean-trypsin-inhibitor, and 50 μg/ml bacitracin], homogenized with a Polytron homogenizer at 900 rpm for 10 strokes, and then ultracentrifuged for 1 h at 4 C at 100,000 × *g*. Membrane pellet was solubilized in a lysis buffer [20 mM HEPES (pH 7.4), 5 mM EDTA, 3 mM EGTA, 150 mM NaCl, and 4 mg/ml dodecyl-B-D-maltoside] for 1 h at 4 C and then ultracentrifuged at 100,000 × *g* for 1 h at 4 C. Glycosylated proteins were purified from the membrane pellet obtained after high speed centrifugation, by wheat-germ agglutinin (WGA) affinity chromatography: the pellet was resuspended in lysis buffer and cycled twice over a 0.5-ml WGA (Vector Laboratories, Inc., Burlingame, CA) column equilibrated with lysis buffer. The column was washed and eluted with lysis buffer containing 3 mM *N*,*N*′,*N*″triacetyl-chitotriose (Sigma-Aldrich Corp., St. Louis, MO).

TABLE 1. Patients' clinical profile at the diagnosis of CD

Patient no.	Sex/age (yr)	Plasma ACTH (pg/ml)	0800 h serum cortisol (μg/dl)	1600 h serum cortisol (μg/dl)	Daily urinary cortisol (μg/24 h)	Serum PRL (μg/liter)	DMX test results[a]	Radiological findings	Surgical findings	Histological findings	Treatment outcome
Group 1											
1	m/60	90.5	17.7	14.2	352.3	4.3	386	No tumor	Diffuse enlargement	Basophilic hyperplasia	Disease persistence
2	f/55	101.6	18.9	20.8	362.4	4.4	443	Microadenoma	Microadenoma	Basophilic adenoma	Cure
3	f/60	247.5	24.3	25.4	622.9	6.8	390	Macroadenoma	Macroadenoma	Chromofobe adenoma	Disease persistence
4	f/33	70.4	25.3	18.9	707.9	4.9	546	Microadenoma	Microadenoma	Chromofobe adenoma	Cure
5	f/33	16.0	25.1	15.3	483.9	4.8	343	Microadenoma	Microadenoma	Basophilic adenoma	Cure
6	m/27	55.1	26.4	26.3	1481.0	6.6	384	Microadenoma	Microadenoma	Basophilic adenoma	Cure
7	f/56	120.4	14.5	16.2	415.6	5.9	633	Macroadenoma	Macroadenoma	Basophilic adenoma	Cure
8	f/35	30.1	31.6	29.1	849.9	5.8	540	No tumor	Diffuse enlargement	Basophilic hyperplasia	Disease persistence
9	f/38	49.0	17.4	11.6	458.4	0.2	220	Microadenoma	Microadenoma	Basophilic adenoma	Cure
10	f/27	49.8	30.7	32.5	1044.2	9.3	553	No tumor	Microadenoma	Basophilic adenoma	Disease persistence
Group 2											
11	f/49	70.0	20.2	20.0	563.0	22.4	124	Microadenoma	Microadenoma	Basophilic adenoma	Cure/disease recurrence
12	f/48	12.6	18.9	19.8	157.9	7.1	88.9	Microadenoma	Microadenoma	Chromofobe adenoma	Cure/disease recurrence
13	f/25	53.0	35.4	28.9	717.7	13.7	444.9	Microadenoma	Microadenoma	Basophilic adenoma	Disease persistence
14	f/35	54.9	24.1	25.5	400.0	9.2	233.5	Microadenoma	Microadenoma	Acidophilic adenoma	Disease persistence
15	m/31	11.0	32.1	20.1	314.5	7.2	99.9	No tumor	Microadenoma	Basophilic adenoma	Disease persistence
16	f/42	82.0	26.8	10.7	254.0	30.1	72.8	No tumor	Diffuse enlargement	Basophilic hyperplasia	Disease persistence
17	m/45	58.9	39.8	27.9	555.9	6.7	245.9	Microadenoma	Microadenoma	Basophilic adenoma	Cure/disease recurrence
18	f/43	107.0	31.9	30.0	431.9	38.9	213.9	Microadenoma	Microadenoma	Chromofobe adenoma	Cure/disease recurrence
19	f/47	89.5	36.0	32.7	316.7	13.0	134.6	Microadenoma	Microadenoma	Basophilic adenoma	Cure/disease recurrence
20	f/26	56.9	36.5	34.5	800.0	15.0	560.9	Microadenoma	Microadenoma	Basophilic adenoma	Cure/disease recurrence

DMX, Dexamethasone; m, male; f, female.

[a] Dexamethasone test, group 1: post-dexamethasone − pre-dexamethasone serum cortisol (change in cortisol); group 2; post-dexamethasone urinary free cortisol levels (micrograms per day).

The protein-containing fractions were determined with the Bradford assay standardized with BSA, pooled, and stored at −80 C. Starting material and WGA-purified membranes proteins were denatured and fractionated under reducing conditions on 12.5% SDS-PAGE, then transferred electrophoretically to Hybond C-Extra nitrocellulose membranes (Amersham Pharmacia Biotech, Oakville, Canada). After transfer, nonspecific binding sites were blocked by Tris-buffered saline-Tween (TBS-T) containing 5% nonfat dried milk. After five washes with TBS-T, membranes were incubated for 16 h at 4 C with a 1:500 dilution of rabbit antihuman D_2 receptor polyclonal antibody (Chemicon International) in TBS-T containing 1% BSA. Membranes were washed five times with TBS-T, then incubated for 1 h at 22 C with 1:1000 dilution of horseradish peroxidase-linked antirabbit IgG (Amersham Pharmacia Biotech) and again washed. The specificity of the antibody was confirmed by preincubating the antibody with the respective immunizing receptor peptide (at a concentration of 100 nM). Immunoreactive bands were detected by the chemiluminescence detection system (ECL Western blot analysis system, Amersham Pharmacia Biotech, Little Chalfont, UK). The immunoreactive bands were visualized by autoradiography after 0.5-min exposure to Biomax film (Eastman Kodak Co.). As the antibody recognizes both the native and the denatured forms of D_2 receptor, bands of 110, 68, and 47 kDa could be visualized by Western blot. The expected band with the procedure used in our laboratory was the 68-kDa denatured form.

RT-PCR

mRNA isolation and cDNA synthesis were carried out according to a previous report (22). mRNA was isolated using Dynabeads oligo(deoxythymidine)$_{25}$ [oligo(dT)$_{25}$; Dynal AS, Oslo, Norway] from a frozen tissue sample. The cells were lysed for 2 min in ice-cold Tris buffer [100 mM Tris-HCl (pH 8), 500 mM LiCl, 10 mM EDTA, 1% lithium dodecyl sulfate (LiDS), 5 mM dithiothreitol, and 5 U/100 μl ribonuclease inhibitor (HT Biotechnology Ltd., Cambridge, UK)]. The mixture was centrifuged at 14,000 rpm for 1 min to remove cell debris. After adding 100 μl prewashed Dynabeads oligo(dT)$_{25}$ to the supernatant, the mixture was incubated for 5 min on ice. Thereafter, the beads were collected with a magnet and washed three times with Tris buffer [10 mM Tris-HCl (pH 8), 0.15 M LiCl, 1 mM EDTA, and 0.1% LiDS] and once with a similar buffer from which LiDS was omitted. mRNA was eluted from the beads in 50 ml of a 2 mM EDTA solution (pH 8) during 2 min at 65 C. To avoid contamination by genomic DNA, the isolated polyadenylated [poly(A)$^+$] mRNA was subjected to a second purification by capturing the RNA on a fresh aliquot of prewashed Dynabeads oligo(dT)$_{25}$ and washing the captured RNA as described above. cDNA was synthesized using the poly(A)$^+$ mRNA captured on the Dynabeads oligo(dT)$_{25}$ in Tris buffer [50 mM Tris-HCl (pH 8.3), 100 mM KCl, 4 mM dithiothreitol, and 10 mM $MgCl_2$] together with 1 mM of each deoxynucleotide triphosphate, 10 U ribonuclease inhibitor, and 2 U avian myeloblastosis virus Super Reverse Transcriptase (HT Biotechnology Ltd.) in a final volume of 20 μl. This mixture was incubated for 1 h at 42 C. One tenth from each cDNA library immobilized on the paramagnetic beads was used for each amplification. The amplification reaction mixture contained cDNA template, 0.5 U SuperTaq (HT Biotechnology Ltd.), 50 μM of each deoxynucleotide triphosphate (HT Biotechnology Ltd.), 5 pmol of each of a pair of oligonucleotide primers specific for human D_1–D_5 receptor subtypes or the hypoxanthine ribosyl transferase (HPRT) in Tris buffer [10 mM Tris-HCl (pH 9), 50 mM KCl, 2 mM $MgCl_2$, 0.01% (wt/vol) gelatin, and 0.1% Triton X-100 in a final volume of 50 μl]. The sequences of the primers for D_1–D_5 and HPRT are listed in Table 2. The PCR reaction was carried out in a DNA thermal cycler (PerkinElmer/Cetus, Gouda, The Netherlands). After an initial denaturation at 94 C for 5 min, the samples were subjected to 40 cycles of denaturation at 94 C for 1 min, annealing for 2 min at 60 C, and extension for 1 min at 72 C. After a final extension for 10 min at 72 C, 10-μl aliquots of the resulting PCR products were analyzed by electrophoresis on 1.5% agarose gels stained with ethidium bromide. Several controls were included in the RT-PCR experiments. To ascertain that no detectable genomic DNA was present in the poly(A)$^+$ mRNA preparation for two DR subtypes, D_1 and D_5, whose genes are intron-less, the cDNA reactions were also performed without reverse transcriptase and amplified with each primer pair. Amplification of the cDNA samples with the HPRT-specific primers served as a positive control for the quality of cDNA. To exclude contamination of the PCR reaction mixtures, the reactions were also performed in the absence of DNA template in parallel with cDNA samples. As a positive control for the PCR reactions of the DR subtypes and HPRT, 0.01 μg human brain cDNA was amplified in parallel with the cDNA samples of each examined pituitary corticotroph tumor.

In vitro functional study

The cell culture was performed according to a previous report (25). Tumor tissue samples were placed in HBSS (Life Technologies, Inc., Paisley, UK), supplemented with 5% human serum albumin (Cealb, CLB, Amsterdam, The Netherlands), penicillin (10^5 U/liter), and fungizone (0.5 mg/liter). After careful removal of blood clots, the samples were minced and washed several times with the HBSS and human serum albumin. The minced tissues were enzymatically dissociated with dispase (1000 U/liter) for 1–2 h at 37 C. After removal of erythrocytes by centrifugation on a Ficoll gradient, the tumor pituitary cells were plated in 24-well plates (Costar, Cambridge, MA) in 1 ml DMEM (Life Technologies, Inc.) culture medium containing 10% (vol/vol) fetal calf serum and penicillin/streptomycin and were incubated at 37 C in a humid CO_2 incubator for 24–48 h to allow them to attach at the bottom of the plate wells. Then, medium was removed and replaced with 1 ml fresh DMEM culture medium, test substances were added, and cells were reincubated at 37 C for 72 h. Afterward, medium was collected and stored at −20 C for the measurement of ACTH secretion. In all experiments, bromocriptine and cabergoline were used as test substances and were added to the cell cultures at concentrations of 0, 10^{-12}, 10^{-10}, 10^{-9}, 10^{-8}, and 10^{-6} M. In two cases (no. 5 and 8), the specificity of the drug effect was tested coincubating the D_2 agonist with an excess (10 μM) of the D_2 antagonist sulpiride.

TABLE 2. Specific oligonucleotide primers for DR subtypes (D_1–D_5) and controls used in the RT-PCR study

Gene		Sequence (5′–3′)	Size of PCR product (bp)
Dopamine receptors			
D_1	Forward	AACACCTCTGCCATGGACG	616
	Reverse	TGATGGCCACAGGGATGTAA	
D_2	Forward	GCGGACAGACCCCACTACAA	521
	Reverse	AAGGGCACGTAGAAGGAGAC	
$D_{2\ short/long}$ isoforms	Forward	CCATGCTGTACAATACGCGCT	$D_{2\ long}$:599;$D_{2\ short}$:512
	Reverse	GGCAATCTTGGGGTGGTCTTT	
D_3	Forward	CCCGCCCACATGCCTACTAT	1106
	Reverse	GAAGGCTTTCCGGAACTCGAT	
D_4	Forward	CCCACCCCAGACTCCACC	259
	Reverse	GAACTCGGCGTTGAAGACAG	
D_5	Forward	ACCTGTGCGTCATCAGCGT	921
	Reverse	TGCGATCGAAAGGACCCTC	
HPRT	Forward	CAGGACTGAACGTCTTGCTC	413
	Reverse	CAAATCCAACAAAGTCTGGCT	

In vivo functional study

The patients were treated with cabergoline. Cabergoline was administered at an initial dose of 1 mg/wk with a monthly increase of 1 mg/wk until normalization of daily urinary cortisol excretion. Plasma ACTH and serum and urinary cortisol levels after 3 months of treatment were compared with the baseline hormonal values. The mean of three different measurements of these hormones on 3 nonconsecutive d of the same week were considered for the baseline and posttreatment evaluations. During cabergoline treatment, blood and urinary samples were collected on the days when the patients did not take the drug. A 50% or greater decrease in daily urinary cortisol excretion was considered a significant clinical response to cabergoline. Moreover, patients who achieved a 50% or greater decrease with normalization of urinary cortisol levels were considered full responders, whereas those who achieved a 50% or greater decrease without normalization of urinary cortisol levels were considered partial responders to cabergoline. Patients who achieved less than a 50% decrease in urinary cortisol levels were considered resistant to cabergoline treatment. After 3 months of treatment, two patients were taking 1 mg, three were taking 2 mg, and the remaining five were taking 3 mg/wk cabergoline.

Hormonal assays

ACTH levels were measured by immunoradiometric assay using a commercially available kit. Serum and urinary cortisol levels were measured by radioimmunological assay, using commercially available kits.

Statistical analysis

Data are expressed as the mean ± SE. The comparison between post- and pretreatment hormone values was made using ANOVA, followed by Newman-Keuls test. The association between parameters was calculated by χ^2 test. Significance was set at 5%.

Results

Histological evaluation and IHC for pituitary hormones

Basophilic adenoma was documented in 12 (60%), basophilic hyperplasia in three (15%), chromofobe adenoma in four (20%), and acidophilic adenoma in one (5%) case. Positive immunostaining for ACTH was documented in all 20 cases (100%) in correspondence with the adenomatous or hyperplasic tissue. Conversely, positive immunostaining for PRL was documented in 13 (65%) cases. However, PRL immunostaining was positive in scattered normal cells (acidophilic ACTH-negative cells) within the tumor tissue in six (30%), in defined areas of normal cells within or around the tumor tissue in four (20%), and in defined areas of tumor tissue (basophilic ACTH-positive cells) in two (10%) cases. The remaining case was characterized by diffuse PRL immunostaining covering all acidophilic ACTH-positive tumor tissues. This case was diagnosed as a mixed ACTH/PRL-secreting pituitary tumor. No significant immunoreactivity was found for GH, TSH, FSH, and LH in all cases; this was only represented by scattered cells around the tumor tissue, probably belonging to contaminating normal pituitary. The histological and immunohistochemical features for pituitary hormones of the tumor samples are listed in Tables 3 and 4 for groups 1 and 2, respectively.

R-LB study

Specific binding of [^{123}I]epidepride was found at autoradiography in eight of 10 (80%) cases in group 1. It was localized in tumor cells and in normal scattered cells or defined areas around tumor tissue or within normal pituitary. The binding was homogeneously distributed in four (50%) and not homogeneously distributed within the tumor tissue in the remaining four (50%) cases. It was scored as moderately positive in three (37.5%) and strongly positive in the remaining five (62.5%) positive cases. The results of the R-LB study are summarized in Table 3. An example of a negative and a positive autoradiography of [^{125}I]epidepride binding are shown in Fig. 1.

Immunoblotting

A specific band of the expected molecular weight (68 kDa) of D_2 receptor was found at the immunoblot of glycoproteins derived from a corticotroph pituitary tumor (Fig. 2).

IHC for D_2 receptor

Specific immunoreactivity for D_2 receptor was found in 15 (75%) cases, eight (80%) in group 1 and seven (70%) in group 2. It was localized in ACTH- and ACTH/PRL-positive tumor cells and in normal PRL-positive cells or areas localized around tumor tissue or within pituitary. The immunoreactivity was homogeneously distributed in seven (46.7%) and was not homogeneously distributed within the tumor tissue in eight (53.3) cases. It was scored as moderately positive in eight (53.3%) and strongly positive in the remaining seven (46.7%) positive cases. In group 1, a complete correspondence was found between the results of R-LB and the IHC studies. The results of IHC are summarized in Tables 3 and 4 for groups 1 and 2, respectively. Examples of a negative and

TABLE 3. DR expression in corticotroph pituitary tumors of patients of group 1

Patient no.	R-LB Study	IHC				RT-PCR study						
		Histology	ACTH	PRL	D2	D1	D2	D2s/1	D3	D4	D5	HPRT
1	+	Basophilic hyperplasia	++	++	+	−	+	Long	−	−	−	+
2	++	Basophilic adenoma	+	−	+	NE	NE	NE	NE	NE	NE	NE
3	−	Chromofobe adenoma	++	−	−	NE	NE	NE	NE	NE	NE	NE
4	+	Chromofobe adenoma	++	+	++	NE	NE	NE	NE	NE	NE	NE
5	+	Basophilic adenoma	++	++	+	NE	NE	NE	NE	NE	NE	NE
6	++	Basophilic adenoma	+	−	++	−	+	Short	−	−	−	+
7	++	Basophilic adenoma	++	++	+	−	+	Long/Short	−	−	−	+
8	−	Basophilic hyperplasia	++	+	−	−	−	−	−	−	−	+
9	++	Basophilic adenoma	++	++	++	NE	NE	NE	NE	NE	NE	NE
10	++	Basophilic adenoma	++	+	++	−	+	Long/Short	−	+	−	+

++, Strongly positive; +, weakly positive; −, negative; NE, not evaluated.

TABLE 4. DR expression in corticotroph pituitary tumors of patients of group 2

Patient no.	IHC				RT-PCR study							*In vitro* ACTH secretion inhibition (%)	
	Histology	ACTH	PRL	D2	D1	D2	D2s/1	D3	D4	D5	HPRT	After bromocriptine	After cabergoline
1	Basophilic adenoma	+	+	+	−	+	Long	−	−	−	+	NE	NE
2	Chromofobe adenoma	+	−	−	−	−	−	−	−	−	+	12	13
3	Basophilic adenoma	++	+	++	−	+	Short	−	−	−	+	NE	NE
4	Acidophilic adenoma	++	++	++	−	+	Long/Short	−	−	−	−	43	57
5	Basophilic adenoma	++	+	−	NE	NE	NE	NE	NE	NE	NE	15	14
6	Basophilic hyperplasia	+	−	+	NE	NE	NE	NE	NE	NE	NE	NE	NE
7	Basophilic adenoma	++	−	++	−	+	Long	−	+	−	+	NE	NE
8	Chromofobe adenoma	++	+	+	−	+	Long	−	−	−	+	53	60
9	Basophilic adenoma	++	+	+	−	+	Long/Short	−	−	−	+	NE	NE
10	Basophilic adenoma	++	−	−	NE	NE	NE	NE	NE	NE	NE	NE	NE

++, Strongly positive; +, weakly positive; −, negative; NE, not evaluated.

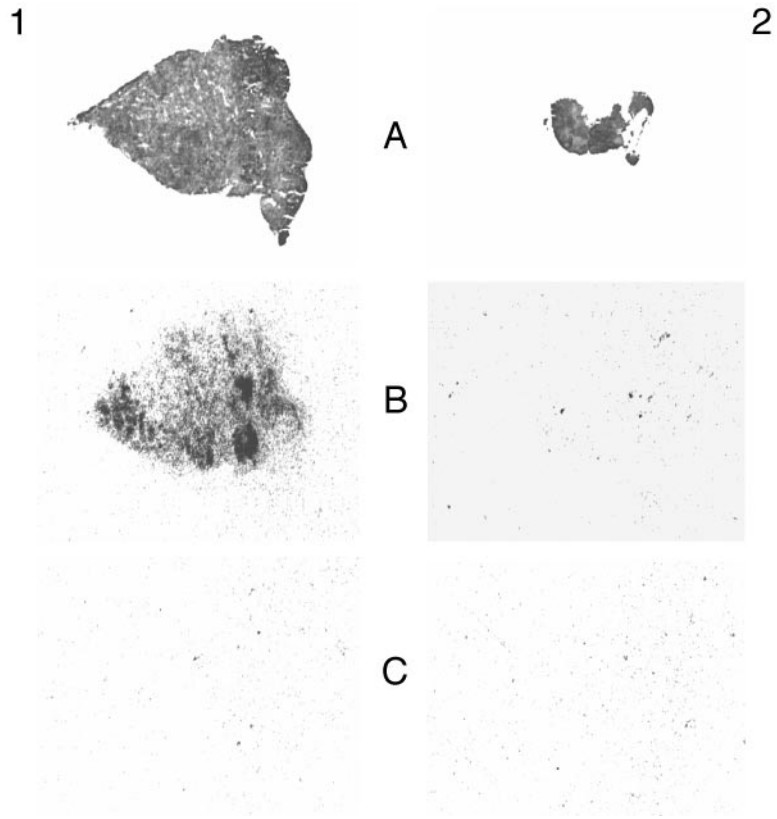

FIG. 1. Expression of D_2 DR subtypes by R-LB study in two cases of human corticotroph pituitary tumors. Photomicrograph of D_2 receptor autoradiography. A, Hematoxylin-eosin-stained section; B, autoradiography showing total binding of [^{125}I]epidepride; C, autoradiography showing nonspecific binding (in the presence of 1 μM cabergoline). The picture shows a positive (no. 1, case 2 of Tables 1 and 3) and a negative (no. 2, case 8 of Tables 1 and 3) case. The specificity of binding is demonstrated by the complete disappearance of the radioactive signal in the presence of an excess of the high affinity D_2 agonist cabergoline.

a positive immunostaining for D_2 receptor are shown in Fig. 3.

RT-PCR study

D_2 receptor was expressed in 10 of 12 cases (83.3%). D_{2long} was found in four (40%), D_{2short} in two (20%), and both D_2 isoforms in four (40%) positive cases. D_4 receptor was expressed in two cases (20%) associated with the expression of D_{2long} in one and with both D_2 isoforms in the remaining case. A complete correspondence was found between the results of RT-PCR and those of R-LB and IHC studies. No expression of other DRs were found. The results of the RT-PCR study are summarized in Tables 3 and 4 for groups 1 and 2, respectively.

In vitro functional study

A significant and dose-dependent inhibition of ACTH secretion was found in two of four cases (50%) in group 2 after both bromocriptine and cabergoline administration. The inhibition rate was 43% in the first case and 52% in the second one after bromocriptine treatment, and 53% in the first case and 60% in the second one after cabergoline administration, respectively. Both cases (100%) had documented expression of D_2 receptor. No significant inhibition of ACTH secretion was found in the remaining two cases after either bromocriptine or cabergoline administration. The inhibition rates were 12% and 15% after bromocriptine and 13% and 14% after cabergoline administration for the two cases, respectively.

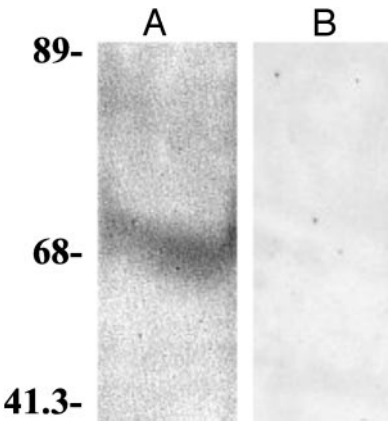

FIG. 2. Immunoblot of glycoproteins derived from a human corticotroph pituitary tumor with D_2 receptor antibody. A and B, Corticotroph pituitary tumor without (A) and with (B) preincubation of the antibody with the antigen. The specificity of the immunoblot is demonstrated by the complete disappearance of the signal after preincubation of the antibody with the antigen (D_2 receptor peptide).

No D_2 expression was found in either case (100%). The specificity of the effects of bromocriptine and cabergoline was demonstrated by the block of this effect in one responsive case by the D_2 antagonist sulpiride (no. 8). No significant effect of sulpiride was demonstrated in one nonresponsive case (no. 5). The results of the *in vitro* study are summarized in Table 4. The individual ACTH responses to bromocriptine and cabergoline in all four corticotroph pituitary tumor cultures are shown in Fig. 4.

In vivo functional study

Significant inhibition of plasma ACTH and serum and urinary cortisol levels after 3 months of cabergoline treatment was found in six of 10 (60%) cases in group 1. However, normalization of urinary cortisol was found in four of 10 (40%) cases, who were considered full responders, whereas a significant decrease without normalization of urinary cortisol was found in the remaining two cases, who were considered partial responders. A significantly different decrease in urinary cortisol levels was found between responder and resistant patients who did not achieve a 50% or greater decrease in urinary cortisol levels after 3 months of cabergoline treatment (79.7 ± 3.6% *vs.* 27.7 ± 7.8%; $P < 0.05$). All cases responsive to cabergoline were associated with D_2 expression in the tumor tissue. Conversely, all cases not responsive to cabergoline were not associated with D_2 expression, with the exception of one case (no. 6 in Tables 1 and 4), which was associated with a poor response to cabergoline treatment. A trend for a significant association between D_2 expression and clinical response to cabergoline ($\chi^2 = 3.35$; $P = 0.067$) was found in the patients in this study. Among the four cases in which a normalization of ACTH and cortisol was obtained, three expressed D_{2short}, which was isolated in one case and associated with D_{2long} in the remaining two cases. The third case was associated with the expression of D_{2long} and D_4. The remaining case with a significant *in vivo* response to cabergoline and the only case not responsive to cabergoline, but D_2 positive, expressed D_{2long}, but not D_{2short} or D_4, receptors. The individual responses of urinary cortisol levels to the 3-month treatment with cabergoline are shown in Figs. 5 and 6.

Discussion

The current study clearly demonstrated D_2 receptor expression in corticotroph pituitary tumors and the effectiveness of cabergoline treatment in controlling the cortisol hypersecretion associated with CD.

CD is a severe chronic disorder resulting from inappropriate and prolonged exposure to excessive endogenous adrenal cortisol secretion secondary to altered pituitary ACTH secretion due to corticotroph pituitary tumor (17); it represents the most common form of chronic endogenous hypercortisolism, accounting for about 80% of cases of Cushing's syndrome (17). Neurosurgical resection of the pituitary tumor is the first-line treatment of CD (26). However, transsphenoidal neurosurgery is successful in 70–80% of cases (27, 28). Moreover, neurosurgical outcome is also affected by the lack of tumor evidence at transsphenoidal exploration in 15% and disease relapse after surgical remission in almost 10% of cases (26–28). Pituitary irradiation and total bilateral adrenalectomy represent second choice treatments in patients not cured by neurosurgery (17). However, although associated with a high success rate, these treatment modalities frequently result in secondary adverse events, such as hypopituitarism in the former and the development of a large and invasive pituitary tumor in the latter. In addition, adrenal insufficiency makes the patient life-long dependent on daily administration of glucocorticoids and mineralocorticoids (17). Medical treatment presently plays a minor role in the treatment of CD; it is usually performed before neurosurgery or after pituitary irradiation to obtain rapid hormonal normalization before the definitive cure (16). Medical treatment is usually performed with adrenal-blocking drugs that do not act at level of the pituitary tumor (16). On the other hand, although several neuromodulatory drugs acting at the pituitary level have been used in the treatment of CD, no single agent has ever demonstrated sufficient effectiveness to achieve widespread clinical use in the management of the disease (29–31).

The only neuromodulatory drug that had a relevant, although controversial, role in the treatment of CD was bromocriptine, the most widely used dopamine agonist. Bromocriptine was hypothesized to induce inhibition of ACTH secretion and/or cell growth in corticotroph pituitary tumors, acting through DRs presumably expressed in the pituitary tumor cells. This hypothesis was based on the evidence that DRs with an inhibitory function were demonstrated to be expressed in PRL-secreting pituitary tumors, where they induced a suppression of hormone secretion and inhibition of tumor growth in a great majority of cases (7–13). In corticotroph pituitary tumors, bromocriptine was found to induce significant inhibition or normalization of cortisol secretion in about 40% of cases after short-term treatment (32). However, controversial results with a success rate ranging from 0–50% were obtained by the different studies that tested the effectiveness of bromocriptine treatment in CD (16). Moreover, normalization of cortisol secretion and/or tumor shrinkage were only sporadically reported after long-

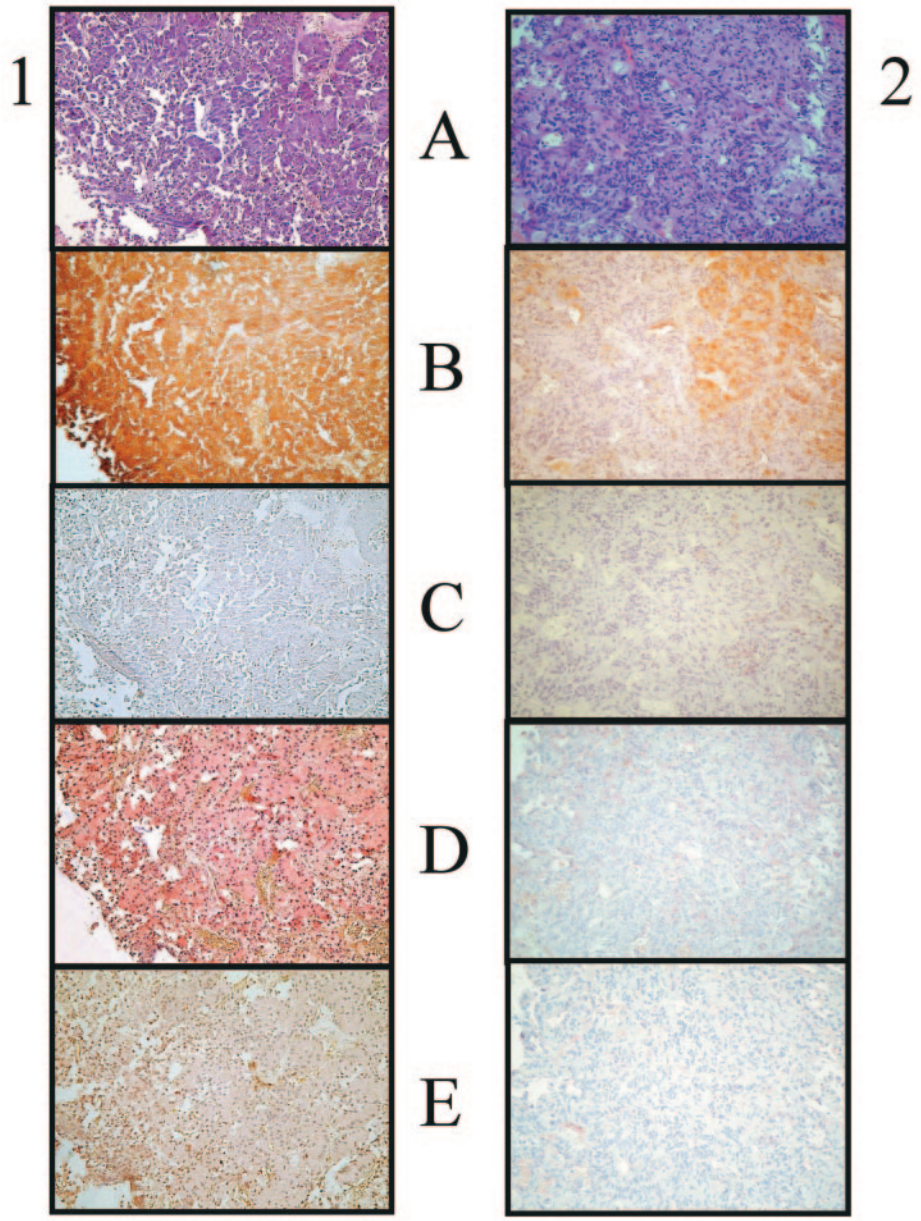

FIG. 3. Expression of D_2 receptor by immunohistochemistry in two cases of human corticotroph pituitary tumors. The immunohistochemical study has been performed on formalin-fixed and paraffin-embedded sections of the tumors. A, Hematoxylin-eosin-stained section; B, ACTH immunostaining; C, PRL immunostaining; D, D_2 receptor immunostaining performed with a specific polyclonal D_2 receptor antibody; E, D_2 receptor immunostaining after preincubation of the antibody with the specific antigen. The picture shows a D_2 receptor-positive (no. 1, case 7 of Tables 1 and 4) and a negative (no. 2, case 5 of Tables 1 and 4) case of ACTH-positive basophilic adenoma. ACTH immunostaining is similar in all cases, whereas PRL immunostaining shows a variable pattern in the different cases. In this figure, PRL immunostaining is negative and scattered positive in cases 1 and 2, respectively. The immunostaining for D_2 receptor shows a homogeneous distribution in half and a dishomogeneous distribution within tumor tissue in the remaining half of the cases. In this figure, it was homogeneously positive and negative in cases 1 and 2, respectively. The specificity of the immunostaining is demonstrated by the disappearance of colorimetric signal after preabsorption of the antibody with 100 nM specific antigen (D_2 receptor peptide). Magnification, ×40.

term treatment with bromocriptine, demonstrating that only a subset of patients with CD was able to respond to chronic bromocriptine treatment (16, 33, 34). On the other hand, cabergoline was reported to induce significant shrinkage of a silent ACTH-secreting pituitary tumor (35), and a functioning ACTH-secreting pituitary tumor developed after bilateral adrenalectomy in a patient cured from CD (36). However, no study has ever evaluated the effectiveness of cabergoline treatment in CD. The current study evaluated the effectiveness of short-term treatment with cabergoline in a group of patients with CD, demonstrating significant inhibition of cortisol secretion in 60% and normalization of cortisol secretion in 40% of cases, suggesting that cabergoline is more effective than bromocriptine and is potentially useful in the treatment of CD.

The biological basis of the effectiveness of dopamine agonists in the treatment of pituitary tumors is represented by DR expression in the tumor. In the pituitary, the response to dopamine agonists is related to the activity of the D_2 receptor (37, 38). This receptor belongs to the family of G protein-coupled receptors and acts through AMP cyclase enzyme inhibition (1, 39). Alternative splicing of the gene encoding D_2 receptor leads to two different isoforms: the short isoform, named D_{2short}, and the long isoform, named D_{2long} (1, 40). In normal human pituitary, D_2 receptor has been demonstrated to be expressed in more than 75% of the cell population, indicating that D_2 receptors are not expressed only in lactotrophs and melanotrophs, which represent no more than 30% of the entire cell population of the normal pituitary gland (37). However, no study has systematically evaluated the colocalization of D_2 receptor with the different pituitary hormones in normal human pituitary gland, leaving only as a likely hypothesis the possible expression of D_2 receptor in non-PRL- and non-MSH-secreting normal pituitary cells. A

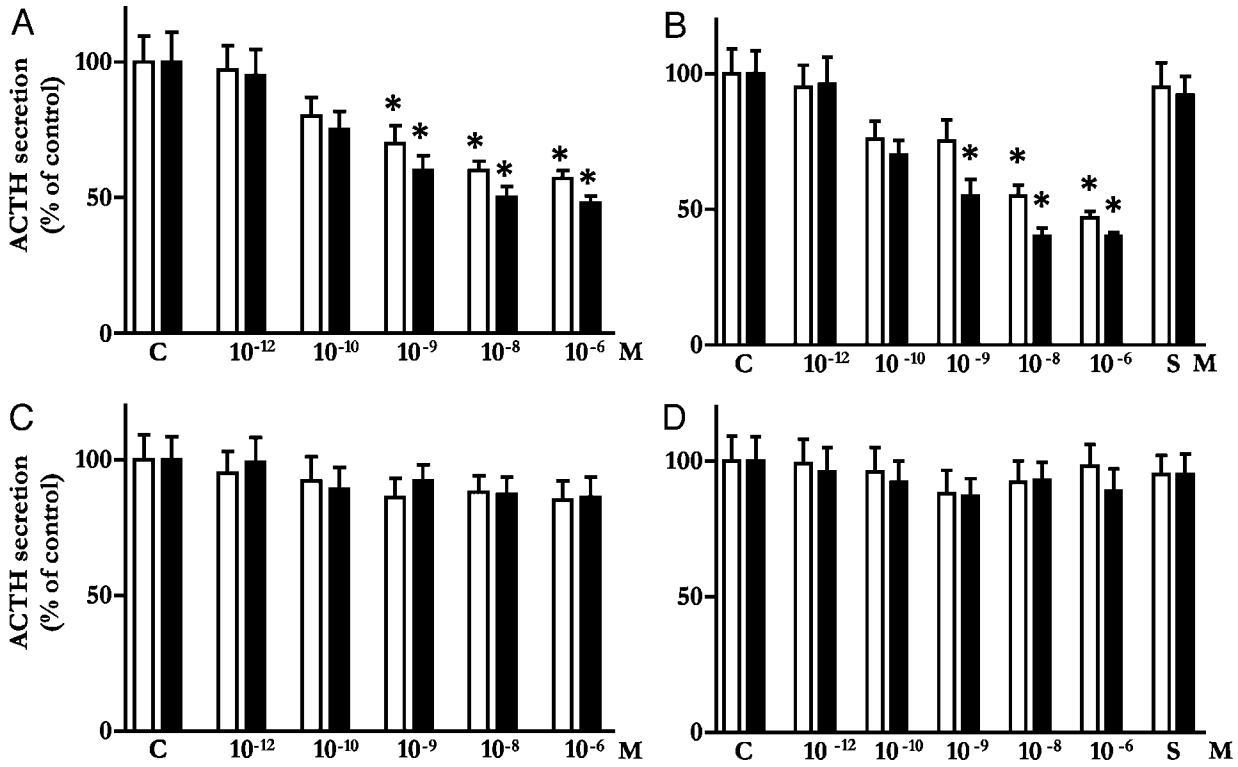

FIG. 4. Effect of *in vitro* administration of bromocriptine (□) and cabergoline (■) on ACTH secretion in cell cultures derived by four different corticotroph pituitary tumors [cases 4 (A), 8 (B), 2 (C), and 5 (D) of Tables 1 and 4]. Corticotroph pituitary tumor cells were incubated in DMEM supplemented with 10% fetal calf serum and penicillin/streptomycin for 72 h in quadruplicate without (C) or with the drugs at concentrations of 10^{-12}, 10^{-10}, 10^{-9}, 10^{-8}, and 10^{-6} M and in two cases (no. 5 and 8) with the drug at a dose of 1 μM coincubated with an excess (10 μM) of the D_2 antagonist sulpiride (S). Values are expressed as the percentage secretion and are the mean ± SEM (n = 4/treatment group).

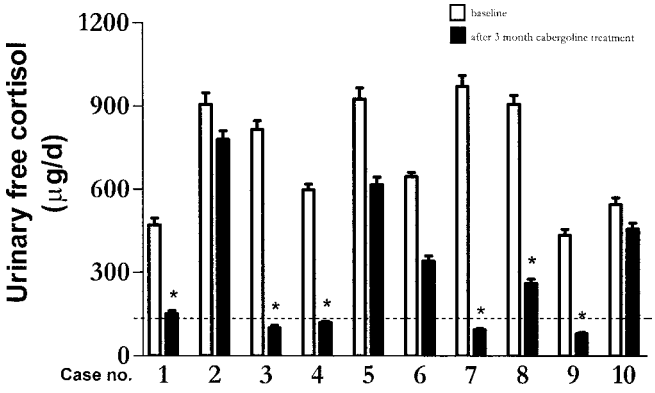

FIG. 5. Effect of *in vivo* administration of cabergoline on urinary cortisol secretion in the 10 patients of group 2. Cabergoline was administered at an initial dose of 1 mg/wk in all patients and at a final dose ranging from 1–3 mg/wk after 3 months of treatment. The dose was increased monthly by 1 mg/wk every month until normalization of urinary cortisol excretion. Daily urinary cortisol is expressed as absolute values in micrograms per day.

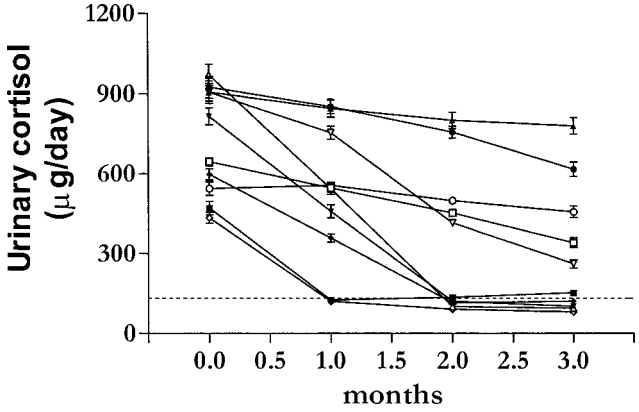

FIG. 6. Individual values of urinary cortisol levels at baseline and during the 3 months of cabergoline treatment of the 10 patients of group 2. Cabergoline was administered at an initial dose of 1 mg/wk in all patients and at a final dose ranging from 1–3 mg/wk after 3 months of treatment. The dose was increased monthly by 1 mg/wk until normalization of urinary cortisol excretion. Daily urinary cortisol is expressed as absolute values in micrograms per day.

variable and heterogeneous expression of D_2 receptor has been recently demonstrated by *in situ* hybridization and IHC in 89% of all types of pituitary tumors, and particularly in 69% of silent or functioning ACTH-secreting pituitary tumors (41). In PRL-secreting pituitary tumors, the presence of a large amount of D_2 receptor explains the good therapeutic response to dopamine agonists, which induces PRL secretion inhibition and tumor shrinkage (42). Moreover, cabergoline has been clearly demonstrated to be more effective than bromocriptine in the treatment of this type of tumor (14, 15). Resistance to dopamine agonists in PRL-secreting pituitary tumor may be explained by the absence or a small amount of D_2 receptor or its functional inactivity (43). D_2 receptor has also been demonstrated in GH-secreting pituitary tumors (44), although their response to dopamine agonists is not as marked

as in PRL-secreting pituitary tumors, probably due to a difference in the abundance or functionality of the receptor (32, 45). However, the relative resistance of these tumors to dopamine agonists was found to be overcome in a percentage of cases with the use of cabergoline (46, 47). In clinically nonfunctioning pituitary tumors, D_2 receptor has been demonstrated in a large number of cases (48, 49), although tumor shrinkage has been reported in only a minority (34, 50). However, in a recent study cabergoline was demonstrated to be more effective than bromocriptine at inducing tumor shrinkage (51). Dopamine agonist resistance in these tumors may be due to the same D_2 receptor abnormalities observed in PRL- and GH-secreting pituitary tumors. However, the expression of D_{2short}, rather than D_{2long}, has been suggested to be associated with a better *in vitro* response to dopamine agonists (52).

As far as corticotroph pituitary tumors are concerned, no definitive evidence is available on D_2 receptor expression, as one study demonstrated the absence of a significant and specific binding of radiolabeled spiperone, a dopamine antagonist, in two corticotroph pituitary tumors (8), whereas another study more recently demonstrated D_2 receptor expression by *in situ* hybridization and IHC studies (41). However, experimental evidence in humans and rats supports the hypothesis of D_2 receptor expression and function in corticotroph pituitary tumors. Indeed, bromocriptine was demonstrated to suppress ACTH secretion from cultured human pituitary tumor cells by a dopaminergic mechanism (53) and to induce apoptosis in AtT-20 cells, a murine corticotroph pituitary tumor cell line (54). The current study represents the first clear demonstration of both D_2 receptor expression and D_2 receptor function in corticotroph pituitary tumors. This finding is strongly supported by the following evidence. 1) D_2 receptor expression has been evaluated at a molecular and cellular levels by different techniques, which permitted demonstration of both transcription of D_2 receptor gene and translation in a D_2 receptor protein. 2) The comparison between the images obtained by R-LB or IHC studies and the histology results clearly demonstrated the presence of D_2 receptors in tumor cell cytoplasm or membranes, excluding that the D_2 receptor demonstrated at a molecular level might exclusively belong to normal pituitary cell infiltration within or normal pituitary tissue surrounding tumor tissue. 3) The *in vitro* effect of dopamine agonists on ACTH secretion inhibition in cell cultures obtained by D_2 positive-corticotroph pituitary tumor samples and the lack of this effect in cell cultures obtained by D_2-negative corticotroph pituitary tumor samples demonstrated the expression of a functional D_2 receptor mediating the effect of dopamine agonists in these tumors. 4) The *in vivo* effect of cabergoline on cortisol secretion inhibition only in CD patients with corticotroph pituitary tumors expressing D_2 receptor definitely confirmed that D_2 receptor may mediate an important effect of dopamine agonists on ACTH and, consequently, cortisol secretion in corticotroph pituitary tumors.

The results of the current study support a possible therapeutic role of dopamine agonists in the treatment of CD. The expression of D_2 receptor in 80% of corticotroph pituitary tumors and the effectiveness of cabergoline treatment in 60%, with normalization of cortisol secretion in 40%, of patients with CD justify the possible use of cabergoline for controlling ACTH and cortisol hypersecretion associated with CD. The different successful rates observed with bromocriptine in past studies and with cabergoline in the present study may be related to the different molecular, biochemical, and pharmacological characteristics of the two dopamine agonists, particularly to the higher specificity and affinity for D_2 receptor and the longer duration of action of cabergoline than bromocriptine (55, 56). The evidence of a similar *in vitro* effect of bromocriptine and cabergoline in the four cases included in the study does not allow us to draw definitive conclusions about the *in vitro* effects of the two drugs in corticotroph pituitary tumors due to the small number of cases evaluated. Moreover, different *in vitro* and *in vivo* behaviors of the two drugs could be also not surprising, considering that the *in vivo* effect of any drug is influenced by several factors that cannot be completely foreseen and reproduced in *in vitro* studies. Finally, it should be mentioned that the CD patients included in the current study were selected from two different endocrine clinics on the basis of the availability of tumor tissue, which excluded the smallest tumors, or on the basis of failure of neurosurgery with consequent persistence of CD and/or recurrence of CD, which mainly included the tumors not easily removable by neurosurgery. In this view, the data presented in the current study do not necessarily represent the whole population of patients with CD. Cabergoline may be added to the list of therapeutic options and drugs that can be adopted in different phases and conditions related to a complex disease such as Cushing's syndrome (57).

In conclusion, the current study demonstrated the expression and function of the D_2 receptor in corticotroph pituitary tumors. The presence of a functional D_2 receptor in 60% and the demonstration of effectiveness of a short-term treatment with the dopamine agonist cabergoline in normalizing ACTH and cortisol secretion in 40% of corticotroph pituitary tumors strongly support the possible therapeutic use of this drug in the management of persistent and/or recurrent CD.

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