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CLINICAL CASE SEMINAR

Intraadrenal Adrenocorticotropin Production in a Case of Bilateral Macronodular Adrenal Hyperplasia Causing Cushing's Syndrome

HERVE LEFEBVRE, CELINE DUPARC, NICOLAS CHARTREL, SYLVIE JEGOU, ANNE PELLERIN, ANNIE LAQUERRIERE, RICHARD IVELL, HUBERT VAUDRY, AND JEAN-MARC KUHN

European Institute for Peptide Research, Department of Endocrinology and Metabolic Diseases, INSERM, U-413 (H.L., J.M.K.), and Department of Pathology, Centre Hospitalo-Universitaire of Rouen (A.P., A.L.), 76031 Rouen, France; Laboratory of Cellular and Molecular Neuroendocrinology, INSERM, U-413, Unité affilieé au Centre National de la Recherche Scientifique, University of Rouen, (H.L., C.D., N.C., S.J., H.V., J.M.K.), 76821 Mont Saint Aignan, France; and Institute for Hormone and Fertility Research, University of Hambourg (R.I.), D-22529 Hambourg, Germany

Adrenochromaffin cells have been shown to physiologically synthesize and secrete ACTH. We have thus hypothesized that excessive intraadrenal ACTH production may be involved in the pathogenesis of primary adrenal Cushing's syndrome. In this report we describe a case of Cushing's syndrome due to bilateral adrenocortical macronodular hyperplasia associated with suppression of plasma ACTH levels. HPLC analysis of adrenal tissue extracts revealed the presence of a peptide coeluting with bioactive ACTH. Immunohistochemical studies showed that ACTH immunoreactivity was detectable in a subpopulation of steroidogenic cells, but not in chromaffin cells. ACTH-positive cells were also labeled by antibodies against relaxin-like factor, a marker of Leydig cells. The presence of ACTH in the hyperplastic tissue resulted from local expression of the gene encoding the ACTH precursor proopiomelanocortin. Finally, hyperplasia fragments, contrary to normal adrenal cortex explants, appeared to release *in vitro* measurable amounts of ACTH. In conclusion, this observation shows that Cushing's syndromes associated with suppressed plasma ACTH levels may be dependent upon ACTH produced within adrenocortical tissue. The term ACTH-independent used to designate primary adrenal Cushing's syndrome may therefore be inappropriate in some cases of bilateral macronodular adrenal hyperplasia with hypercortisolism and undetectable plasma ACTH levels. (*J Clin Endocrinol Metab* 88: 3035–3042, 2003)

ACTH-INDEPENDENT CUSHING'S syndrome results from cortisol overproduction by adrenocortical tumors or, more rarely, by bilateral macronodular adrenal hyperplasias. The mechanisms responsible for the maintenance of cortisol secretion in the absence of plasma corticotropin have long remained unknown. Over the last decade, several groups have demonstrated that apparently autonomous cortisol production may be under the control of abnormal membrane receptors in both adrenal tumors and bilateral hyperplasias with Cushing's syndrome (1). These aberrant receptors include ectopic receptors for gastric inhibitory polypeptide, LH, or catecholamines and abnormally active eutopic receptors, such as vasopressin V_1 and serotonin 5-hydroxytryptamine$_4$ (5-HT$_4$) receptors (1). Arginine vasopressin and 5-HT$_4$ are physiologically present in the adrenal cortex and may, therefore, stimulate cortisol secretion from adrenocortical lesions through a paracrine mode of regulation (2–4). In agreement with this hypothesis, intensive studies have provided strong evidence that corticosteroid secretion from normal adrenal gland is regulated by multiple intraadrenal factors, including bioactive peptides and classical neurotransmitters, which are released in the vicinity of steroidogenic cells by chromaffin cells, nerve endings, cells of the immune system, or endothelial cells (5, 6). In particular, ACTH itself has been shown to be locally synthesized and released by adrenochromaffin cells (7–9). This new physiological concept suggests that, in some cases of primary adrenal Cushing's syndrome, excessive cortisol secretion and adrenocortical cell proliferation may be the result of an overproduction of intraadrenal paracrine stimulatory factors.

In this report we describe a case of bilateral adrenocortical macronodular hyperplasia causing Cushing's syndrome associated with intraadrenal overproduction of ACTH. ACTH was detected in adrenocortical tissue extracts, and ACTH immunoreactivity was observed in a subpopulation of steroidogenic cells, but not in chromaffin cells. The presence of ACTH in the adrenocortical tissue resulted from local expression of the gene encoding the ACTH precursor proopiomelanocortin (POMC). Finally, the hyperplastic tissue, but not normal adrenal cortex explants, appeared to release *in vitro* measurable amounts of ACTH.

Abbreviations: 5-HT$_4$, 5-Hydroxytryptamine$_4$; Pit1, pituitary-specific transcription factor-1; POMC, proopiomelanocortin; Prop1, prophet of pituitary-specific transcription factor-1; Ptx1, pituitary homeobox factor-1; RLF, relaxin-like factor.

Subjects and Methods

Patient

A 46-yr-old man presented with a history of weight gain, muscle weakness, and hypertension resistant to tritherapy with furosemide,

spironolactone, and atenolol. Physical examination revealed central obesity (92.6 kg, 177 cm), skin changes including epidermal atrophy and abdominal purple striae, and proximal amyotrophy of the lower limbs. His resting pulse was 72 beats/min, and his mean blood pressure was 184/108 mm Hg. Plasma cortisol concentrations were 806 nmol/liter [29.2 μg/dl; normal, 250–850 nmol/liter (9.1–30.8 μg/dl)] in the morning and 759 nmol/liter (27.5 μg/dl) in the evening. Urinary cortisol excretion was 826 nmol/d [300 μg/d; normal, 55–220 nmol/d (20–80 μg/d)]. The morning plasma ACTH concentration was undetectable [<1.1 pmol/liter (5 pg/ml); normal, 2.2–17.6 pmol/liter (10–80 pg/ml)]. Plasma and urinary cortisol values did not significantly decrease in response to high doses (8 mg/d for 2 d) of dexamethasone. Administration of cosyntropin (250 μg, iv) induced a substantial increase in plasma cortisol levels from 909 nmol/liter (32.9 μg/dl) to 2455 nmol/liter (88.9 μg/dl). Computed tomography revealed bilateral macronodular adrenal hyperplasia with nodules measuring up to 2.5 cm on the right and 3.5 cm on the left. Potentially aberrant membrane hormone receptors were systematically searched for, after informed consent of the patient, using a clinical protocol previously described (10). The study was approved by the regional ethics committee. Briefly, plasma cortisol levels were measured in response to a posture test, a standard mixed meal, 100 μg GnRH, iv; 100 μg TRH, iv; 1 mg glucagon, iv; and 10 mg, orally, of the 5-HT_4 receptor agonist cisapride (Prepulsid, Janssen Pharmaceuticals-Cilag, Boulogne-Billancourt, France). The adrenal sensitivity to arginine vasopressin was not investigated because of severe hypertension. Plasma cortisol and ACTH were measured using a commercial immunoluminescent kit (Immulite 2000 Cortisol, Diagnostics Products, Los Angeles, CA) and an immunoradiometric assay (Nichols Institute Diagnostics, San Juan Capistrano, CA), respectively. For all tests, a positive cortisol response was arbitrarily defined as a 25% increase in plasma cortisol. Posture and cisapride tests induced a significant increase in plasma cortisol levels reaching, respectively, +44% and +49% without any variation in plasma ACTH levels, which remained undetectable throughout the study. None of the other tests gave rise to a positive cortisol response. The patient was treated with o'p'DDD (mitotane, 3 g, orally, three times daily). Two months later, clinical evaluation showed no improvement of the symptoms, and urinary free cortisol remained elevated [1109 nmol/d (402 μg/d)]. o'p'DDD was then substituted by ketoconazole (200 mg, orally, three times daily), which induced a significant regression of clinical signs of hypercortisolism and normalization of urinary free cortisol [139 nmol/d (50.4 μg/d)] after 2 wk. The patient underwent bilateral adrenalectomy 1 month after the beginning of ketoconazole therapy and was postoperatively substituted orally with hydrocortisone (30 mg/d) and fluorohydrocortisone (50 μg/d). All clinical and biological signs of Cushing's syndrome completely resolved after removal of the adrenal glands.

Fragments of the two hyperplastic adrenal glands were obtained at surgery and either immediately transported to the laboratory in culture medium for perifusion experiments, frozen on dry ice, and stored at –80 C until HPLC, *in situ* hybridization, and RT-PCR experiments or fixed in formalin and embedded in paraffin for immunohistochemical studies (see below). Normal adrenal tissue explants (control tissues) were obtained from patients undergoing expanded nephrectomy for kidney cancer. The protocol of collection of the tissues and the experimental procedures were approved by the regional ethics committee, and written informed consent was obtained from all subjects.

Biochemical characterization of ACTH-like immunoreactivity

An adrenocortical fragment (2.5 g wet weight) was immersed for 15 min in 0.5 M boiling acetic acid. The tissue was homogenized using a glass Potter and centrifuged (6000 × *g*, 30 min, 4 C). The supernatant was prepurified on Sep-Pak C_{18} cartridges (Waters Corp., Milford, MA), and the eluate was evaporated under reduced pressure. The dried extract was resuspended in water/trifluoroacetic acid (99.9:0.1, vol/vol) and analyzed by HPLC on a 0.46 × 25-cm Vydac 219TP54 diphenyl column (Separations Group, Hesperia, CA) equilibrated with a solution of acetonitrile/water/trifluoroacetic acid (14.0:85.9:0.1, vol/vol/vol) at a flow rate of 1 ml/min. The concentration of acetonitrile in the eluting solvent was raised to 42% over 40 min. Fractions were collected every minute, and ACTH-like immunoreactivity was determined by an immunoradiometric assay (Nichols Institute Diagnostics, San Juan Capistrano, CA). Synthetic human ACTH-(1–39) and ACTH-(1–24) were used as reference peptides and chromatographed in the same conditions as the tissue extracts.

Immunohistochemical studies

Deparaffinized sections of the hyperplastic tissue were incubated with monoclonal mouse antibodies to ACTH (1:500; DAKO Corp., Trappes, France), monoclonal mouse antibodies to chromogranin A (1:300; DAKO Corp.), polyclonal rabbit antibodies to 17α-hydroxylase (1:100; provided by Drs. V. Luu The and G. Pelletier, Laval University Medical Center, Québec, Canada), and polyclonal rabbit antibodies to the Leydig cell marker relaxin-like factor (RLF; 1:2000) (11) according to the procedure previously described (12). Bound antibodies were detected by the linked streptavidin-biotin-peroxidase method (DAKO Corp.), and the enzyme reaction was visualized with 3-amino-ethylcarbazole (DAKO Corp.). For double labeling of tissue slices with antibodies against ACTH and 17α-hydroxylase, the sections were stained with both fluorescein isothiocyanate-conjugated goat antimouse γ-globulins (1:100; Nordic Immunology Laboratories, Tilburg, The Netherlands) and Texas Red-conjugated donkey antirabbit γ-globulins (1:100; Nordic Immunology Laboratories) and examined on a confocal laser scanning microscope (Leica Corp., Heidelberg, Germany). Specificity controls of the immunohistochemical reactions were performed by incubating adjacent sections with the ACTH antiserum preabsorbed with synthetic ACTH and/or replacement of the RLF antiserum with preimmune serum of the same rabbit used at the same dilution.

In situ hybridization

Sense and antisense riboprobes were prepared by *in vitro* transcription of a 409-bp fragment of the rat POMC gene exon III (position 221–629) subcloned in PCR II, in the presence of digoxigenin-11-UTP. Tissue sections (14 μm) were delipidized in chloroform, fixed in 4% paraformaldehyde, acetylated, treated with Triton X-100 (0.2%), and hybridized overnight at 55 C as previously described (13). Specific labeling was visualized by incubation of the sections with an antidigoxigenin antiserum conjugated to alkaline phosphatase, followed by the chromogen solution (4-nitro blue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate).

RT-PCR

Total RNA from the hyperplastic adrenal cortex, a somatotropic adenoma, and three normal adrenal glands was extracted by the acid guanidium-thiocyanate-phenol-chloroform procedure using Tri-Reagent (Sigma-Aldrich Corp., St. Louis, MO). The concentration of total RNA was determined by measuring OD at 260 nm. Total RNA (1 μg) from each tissue was converted into single-stranded cDNA using Superscript II (Life Technologies, Inc., Eragny, France) with oligo(deoxythymidine)$_{12–18}$ primer (0.5 μg/μl). Amplification of the

TABLE 1. RT-PCR analysis of pituitary marker mRNAs

Primer	Primer sequence (5′-3′)	Product (bp)	Cycles	Ta
Ptx1				
s	CTAGAGGCCACGTTCCAGAG	439	40	60
as	CGGTGAGGTTGTTGATGTTG			
p	AGTCCATGTTCTCAGCACCC			
Prop1				
s	AACCAGTACCCCGACATCTG	202	40	60
as	TGCGTAAGAATAGGGGCAAG			
p	TCCAGGTCTGGTTCCAGAAC			
Pit1				
s	TTCTGACGCCTCTGCAACTCT	698	40	51
as	CAGCCATCCTCATGATCTCT			
p	AACCTATGGAGTGATGGCAG			
GAPDH				
s	TGCTGAGTAYGTCGTGGAGTC	191	40	58
as	TTGGTGGTGCAGGAKGCATTGC			

Oligonucleotide sequence for sense (s), antisense (as), and hybridization probes (p) are shown. Ta, Annealing temperature.

cDNAs encoding the pituitary markers pituitary homeobox factor-1 (Ptx1), pituitary-specific transcription factor-1 (Pit1) and prophet of Pit1 (Prop1) was performed by PCR using specific primers (Table 1). Two other primers (5′-TGCTGAGTAYGTCGTGGAGTC-3′ and 5′-TTGGTGGTGCAGGAKGCATTGC-3′), corresponding to bases 297–317 and 467–488, respectively, of the cloned glyceraldehyde-3-phosphate dehydrogenase sequence were used for semiquantitation of reverse transcribed mRNAs. The amplified products were analyzed in 1.5% agarose gels, blotted on a nylon membrane, and hybridized with a [^{32}P]ATP-labeled internal oligonucleotide (Table 1).

Perifusion studies

ACTH secretion from the patient's adrenal hyperplasia tissue and from three normal adrenocortical explants obtained from patients undergoing expanded nephrectomy for kidney cancer was investigated *in vitro* using a perifusion system technique previously described (3). The protocol of collection of the tissues and the experimental procedures were approved by the regional ethics committee. In all cases adrenal tissue was obtained at surgery after obtaining informed consent from the patients. Hyperplasia and/or normal adrenal gland explants were immediately transported to the laboratory in DMEM. The adrenal cortex was carefully dissected from the medulla and diced into 1-mm^3 fragments. The tissues were mixed with Bio-Gel P_2 and transferred into perifusion columns (~200 mg wet tissue/column). The perifusion columns were supplied with DMEM at a constant flow rate (300 μl/min). pH (7.4) and temperature (37 C) were kept constant throughout the experiment. The perifusion medium was continuously gassed with a 95% O_2-5% CO_2 mixture. Fractions of the effluent perifusate were collected every 5 min and immediately frozen at −80 C until assay. ACTH concentrations were measured in the fractions using the immunoradiometric assay. The standard curve of the assay was established by diluting synthetic human ACTH in DMEM. Aliquots of the effluent perifusates were all assayed for ACTH at the same time. Cortisol levels were determined in the same fractions by RIA, as previously described (3).

Results

Histological examination

Microscopic examination of adrenal tissue revealed the presence of multiple nodules of cortical hyperplasia distributed in the two adrenal glands. The nodules were constituted by spongiocytic cells with no criteria of malignancy. No territory of necrosis was observed. The internodular steroidogenic tissue was not atrophic.

Biochemical characterization of ACTH-like immunoreactivity

Characterization of the ACTH contained in Sep-Pak-prepurified adrenocortical extracts was carried out by combining reverse phase HPLC with immunoradiometric detection. The immunoreactive material eluted as a single peak with a retention time of 24 min (Fig. 1). Synthetic human ACTH-(1–39), chromatographed under the same conditions, coeluted with the endogenous peptide (retention time, 24.3 min), whereas ACTH-(1–24) was resolved at 16.1 min (Fig. 1). The adrenal content of immunoreactive ACTH was 436 fmol (1.98 ng)/g wet tissue. Its partial release in the interstitial space may produce a local concentration of 5 nmol/liter (22 μg/liter), according to the method of calculation provided by Mazzocchi *et al.* (14).

Immunohistochemical studies

Labeling of tissue slices with the ACTH antibodies revealed the presence of clusters of immunoreactive cells located at the periphery and in the central zone of the hyperplastic nodules (Fig. 2A). ACTH-positive cells had the morphological characteristics of spongiocytic cells, *i.e.* abundant cytoplasm with numerous lipid droplets. In some of these cells the immunoreactivity was restricted to a limited area of the cytoplasm (Fig. 2B). Preincubation of the ACTH antiserum with synthetic ACTH totally abolished immunostaining (data not shown). No ACTH immunoreactivity was detected in the medulla and intracortical medullary rays (Fig. 3, A and B). Spongiocytic-like ACTH-immunopositive cells were chromogranin A negative, but were labeled by antibodies against 17α-hydroxylase (Fig. 4, A and B) and antibodies against RLF, a specific marker of Leydig cells (Fig. 5,

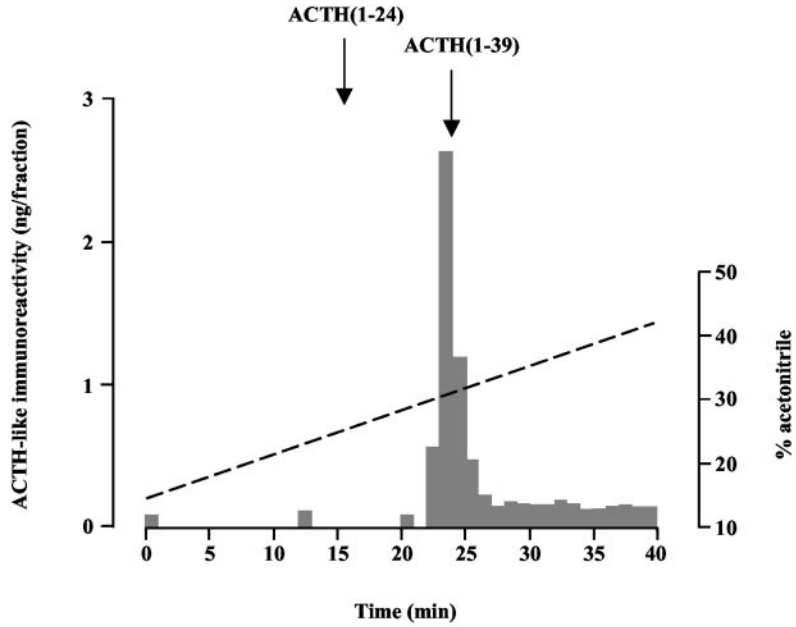

FIG. 1. Reverse phase HPLC analysis of ACTH in the adrenocortical hyperplastic tissue. A Sep-Pak-prepurified adrenocortical hyperplasia extract was chromatographed on a Vydac diphenyl column, and the immunoreactive material contained in the eluting fractions was quantified by immunoradiometric assay. The *dashed line* shows the concentration of acetonitrile in the eluting solvent. The *arrows* indicate the retention times of synthetic human ACTH-(1–39) and ACTH-(1–24).

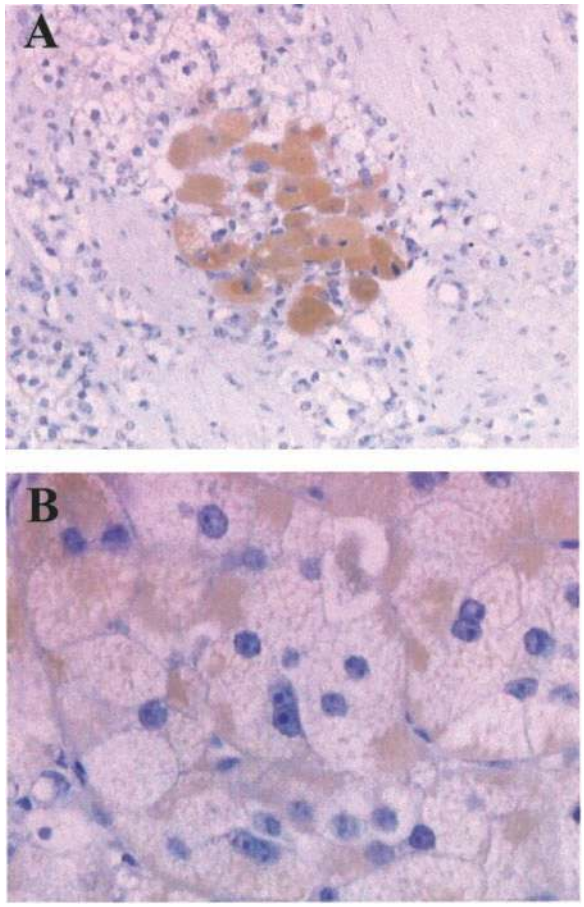

FIG. 2. Immunohistochemical localization of ACTH in the adrenocortical hyperplastic tissue. A, A small group of ACTH-positive cells in the central region of a nodule of the hyperplasia (magnification, ×120). These cells contain large lipid droplets, and the labeling is often restricted to a limited area of the cytoplasm (B; magnification, ×200).

A and B). Substitution of RLF antibodies with immunoglobulins obtained in the same animal before immunization resulted in complete loss of the immunoreaction (Fig. 6, A and B). In addition, no RLF immunoreactivity was observed in a normal adrenal gland section (Fig. 6C).

In situ hybridization

An intense hybridization signal revealing the presence of POMC mRNA was detected in a subpopulation of cells of the tissue (Fig. 7, A and B). The distribution of the signal was similar to that of ACTH immunoreactivity. No specific hybridization signal was observed when adjacent sections were incubated with the sense riboprobe instead of the antisense riboprobe (Fig. 7C).

RT-PCR

The occurrence of mRNAs encoding, respectively, the pituitary markers Pit1, Ptx1, and Prop1 was investigated by RT-PCR amplification in the patient tissue, a pituitary somatotropic adenoma, and three normal adrenal glands. Pit1, Ptx1, and Prop1 mRNAs were detected in the pituitary adenoma, but not in the patient tissue or the normal adrenal explants (Fig. 8).

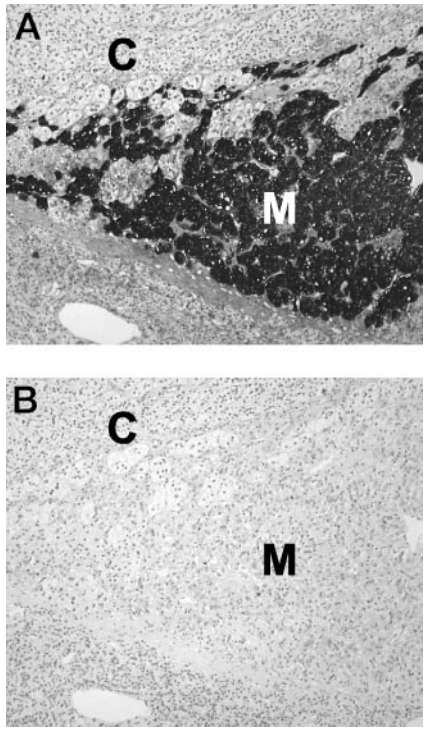

FIG. 3. Labeling of consecutive sections of the medulla with antibodies against chromogranin A and ACTH. A, Intense labeling of the medullary tissue by antibodies to chromogranin A (magnification, ×70). B, No detectable immunoreactive material was observed in adrenochromaffin cells after incubation of a consecutive section of the tissue with antibodies to ACTH (magnification, ×70). M, Medulla; C, cortex.

Perifusion studies

Fragments of the adrenocortical hyperplasia explants or from three normal adrenal cortexes were perifused as described in *Subjects and Methods*. Perifused hyperplasia fragments released measurable amounts of ACTH (Fig. 9). The concentration of ACTH in the effluent perifusate spontaneously fluctuated between 2.90 and 4.99 pmol/liter (13.2 and 22.7 pg/ml) and was significantly correlated with cortisol concentration ($r^2 = 0.27$; $P < 0.001$). The mean secretion rate of ACTH was 4.34 ± 0.86 (±SD) fmol (19.7 ± 3.93 pg)/g wet tissue·min. In contrast, ACTH was not detectable in the effluent perifusate of normal adrenal gland explants (Fig. 9). Normal adrenal tissue remained functional throughout the study, as shown by the substantial levels of cortisol [ranging from 99.4–615 pmol/liter (36.0–223 ng/liter)] measured in the perifusate.

Discussion

Recent studies have shown that in the mammalian adrenal gland corticosteroidogenesis is not only regulated by circulating corticotropic factors, but is also influenced by various bioactive peptides produced in the vicinity of adrenocortical cells (5, 6). In particular, adrenochromaffin cells have been shown to stimulate corticosteroid secretion through local

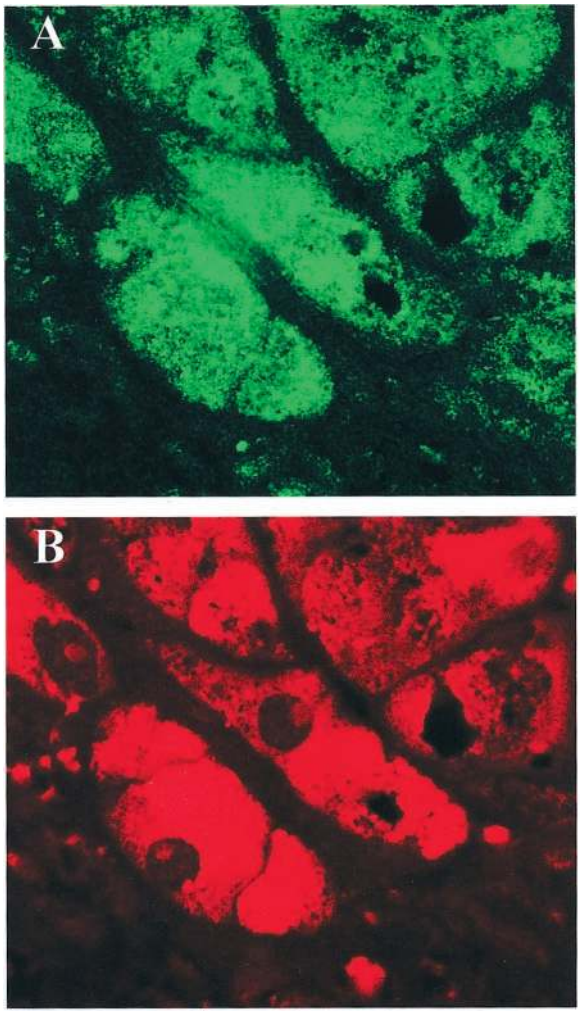

FIG. 4. Dual channel confocal laser scanning microscopic analysis of a group of adrenocortical cells labeled with antibodies against ACTH and 17α-hydroxylase. A, Group of ACTH-positive cells within the hyperplastic cortex. The immunoreactivity was revealed with fluorescein isothiocyanate-conjugated goat antimouse γ-globulins (magnification, $\times$675). B, The same ACTH-positive cells were also labeled by a rabbit polyclonal antibody to 17α-hydroxylase. The immunoreactivity was revealed with Texas Red-conjugated donkey antirabbit γ-globulins (magnification, $\times$675).

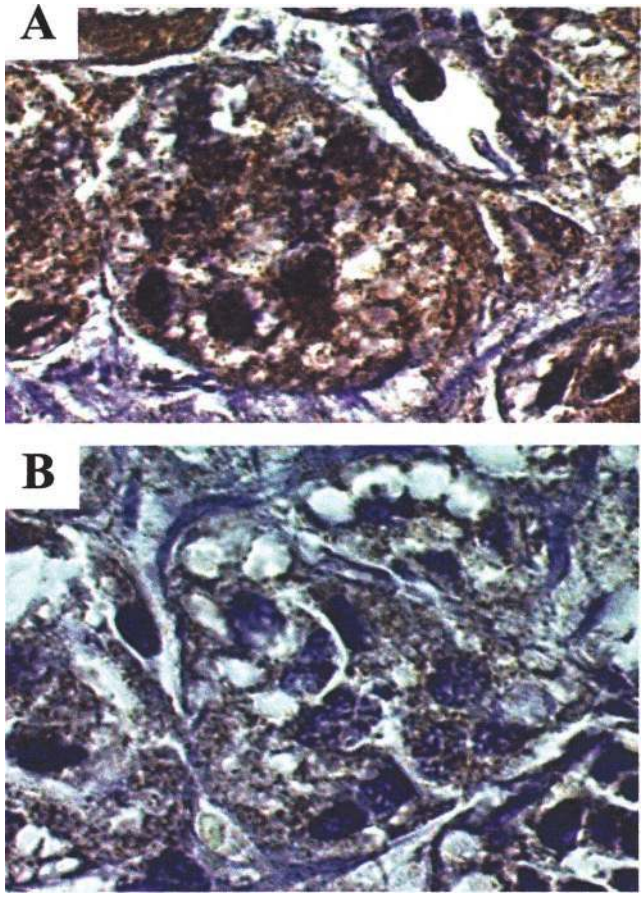

FIG. 5. Labeling of consecutive sections of the hyperplasia tissue with antibodies against ACTH and RLF. A, Group of spongiocytic-like ACTH-positive cells located at the periphery of a hyperplastic nodule in the subcapsular region of the cortex (magnification, $\times$1200). B, The same cells were also labeled by the antibodies against RLF (magnification, $\times$1200).

release of ACTH (15, 16). We have thus hypothesized that intraadrenal production of ACTH could be involved in the pathogenesis of primary adrenal Cushing's syndrome due to bilateral macronodular adrenocortical hyperplasia. In the present report we provide evidence for the occurrence of a mature form of immunoreactive ACTH coeluting with synthetic ACTH-(1–39) in extracts of adrenocortical hyperplasia tissue removed from a patient with Cushing's syndrome. Labeling of the adrenal hyperplasia tissue with a specific ACTH antibody revealed the presence of clusters of immunoreactive cells exhibiting the morphological characteristics of steroidogenic cells. Consistent with this observation, ACTH-immunopositive cells were also labeled by antibodies against 17α-hydroxylase, a key enzyme in the synthesis of cortisol and androgens. In contrast to previous results obtained in an adrenocortical-pituitary hybrid tumor causing Cushing's syndrome (17), RT-PCR experiments failed to detect mRNAs encoding the pituitary markers Pit1, Ptx1, and Prop1. ACTH concentrations were apparently much higher in steroidogenic cells labeled by the ACTH antibody than in the medullary tissue, where no ACTH immunoreactivity was detected with the technique used in the study. The intense expression of POMC mRNA in ACTH-positive cells indicates that the presence of corticotropin in the hyperplastic adrenal cortex can be ascribed to local biosynthesis. Ectopic expression of the POMC gene by steroidogenic cells was responsible for intraadrenal overproduction of ACTH, as demonstrated by the perifusion data, which showed that the hyperplastic tissue released much larger amounts of ACTH than normal adrenal tissue. Several lines of evidence indicate that the intraadrenal overproduction of ACTH was probably the cause of the patient's adrenal hyperplasia and hypercorticism: 1) the nodules of hyperplasia were organized around clusters of ACTH-containing cells; 2) the peptide analyzed in the tissue extracts had the same chromatographic characteristics as bioactive ACTH; 3) the concentration of ACTH in the intraadrenal interstitial space estimated from the measurement of adrenal content was 1000 times greater than the normal plasma levels of the peptide; 4) ACTH and cortisol

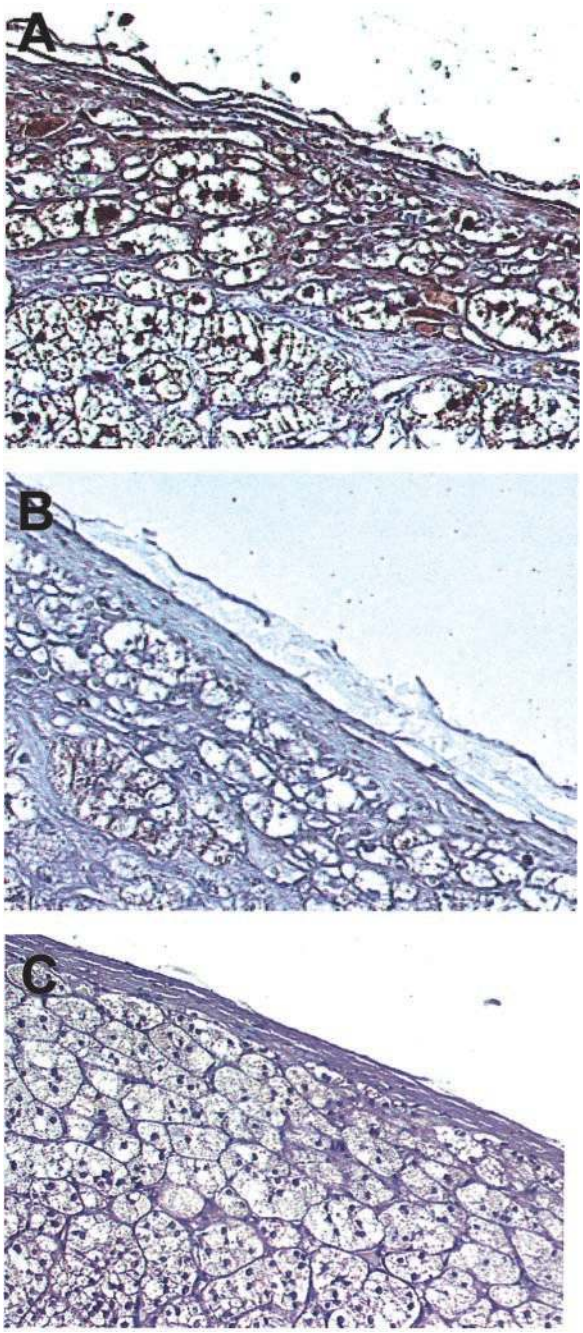

FIG. 6. Labeling of hyperplasia tissue and normal human adrenal cortex with antibodies against RLF. A, Groups of RLF-positive cells located at the periphery of a hyperplastic nodule in the subcapsular region of the cortex (magnification, ×300). B, Incubation of a consecutive section of the hyperplasia tissue with preimmune rabbit serum resulted in complete loss of the immunoreaction (magnification, ×300). C, Section of a normal human gland labeled with the RLF antibodies showing the absence of staining (magnification, ×160).

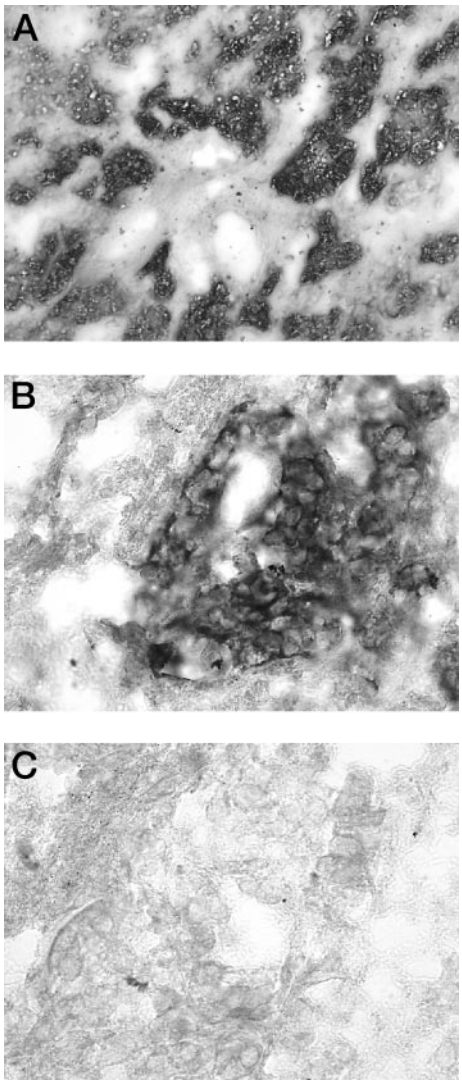

FIG. 7. Localization of POMC gene expression by *in situ* hybridization in the adrenocortical hyperplastic tissue. A, Clusters of cells stained by the digoxigenin-11-UTP-labeled POMC antisense riboprobe. The hybridization reaction was visualized by incubating the section with an antidigoxigenin antiserum conjugated to alkaline phosphatase revealed with the chromogen solution (magnification, ×120). B, Close-up view of a cluster of cells labeled by the POMC antisense riboprobe (magnification, ×350). C, No hybridization signal was observed in the same region of the tissue after incubation with the POMC sense riboprobe (magnification, ×350).

levels measured in the elution medium from perifused hyperplastic adrenocortical tissue were significantly correlated; and 5) the adrenocortical tissue was extremely sensitive to the stimulatory action of ACTH, as shown by the results of the *in vivo* cosyntropin test. The contrast between the high intraadrenal ACTH levels and the low concentration of the peptide in the perifusate indicates that ACTH was rapidly and actively metabolized *in situ* after binding to its receptor on adrenocortical cells. This observation also explains why intraadrenal production of ACTH was not sufficient to elevate plasma ACTH levels, as ACTH secretion by the pituitary gland was suppressed by the chronic increase in plasma cortisol levels. The pathological cortisol response to posture and cisapride tests suggests the occurrence of aberrantly expressed membrane receptors in the adrenal tissue. These ectopic and/or overexpressed eutopic receptors may have contributed to the maintenance of active steroidogenesis in the absence of circulating ACTH. However, the high local production of intact ACTH is sufficient to explain the hypercortisolism.

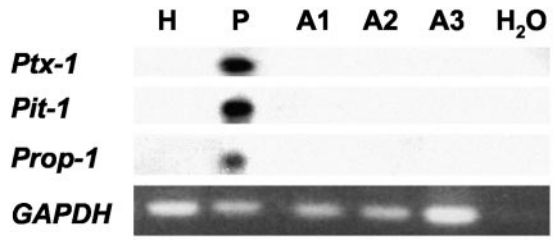

FIG. 8. RT-PCR analysis of mRNA encoding the pituitary markers Pit1, Ptx1, and Prop1 in the patient's adrenal hyperplasia (H), one pituitary somatotropic adenoma (P) and three normal adrenal glands (A1, A2, and A3). Specific primers for Pit1, Ptx1, and Prop1 were used to amplify fragments of 698, 439, and 202 bp, respectively.

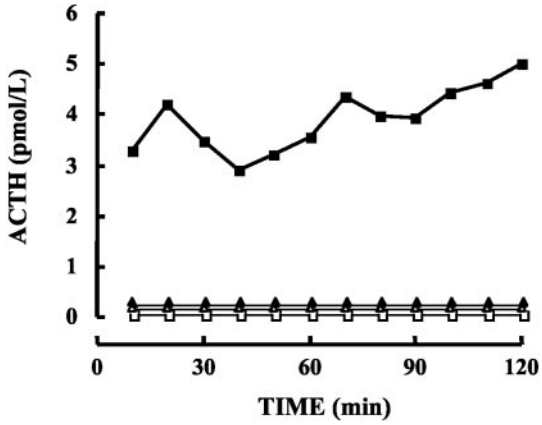

FIG. 9. Corticotropin release by perifused adrenocortical explants obtained from the case patient (■) and three normal adrenocortical fragments (▲, △, and □). After a 120-min equilibration period, tissue explants (~200 mg wet tissue/column) were perifused with DMEM warmed at 37 C. ACTH levels were measured in each 5-min fraction by immunoradiometric assay. Each *point* represents the mean ACTH production of two consecutive 5-min fractions. To convert the values for ACTH to picograms per milliliter, multiply by 4.55.

The mechanisms by which a subpopulation of steroidogenic cells in the tissue abnormally produced ACTH remain unknown. ACTH-immunoreactive cells may correspond to Leydig-like cells, as it is clearly demonstrated that Leydig cells, in addition to their steroidogenic activity, physiologically synthesize POMC and its derived peptides (18). It is classically considered that adrenocortical and Leydig stem cells both derive from the mesonephros; immature Leydig cells then migrate to the gonads (19). We propose that in the present case, some Leydig stem cells may have not migrated to the gonads during embryogenesis, explaining the intermingling of normal adrenocortical cells with ACTH-immunoreactive steroidogenic cells in the two adrenal glands of the patients. Gradual expression of the POMC gene by Leydig-like cells may have further led to nodular hyperplasia of the cortex and cortisol overproduction. This hypothesis is strongly supported by the immunohistochemical data showing that ACTH-positive cells are labeled by antibodies directed against RLF, which is, in males, a highly specific marker of Leydig cells (11). In contrast, RLF immunoreactivity could not be detected in ACTH-negative tissue or in a section of a normal human adrenal gland. This latter observation is consistent with previous results indicating that RLF is not physiologically expressed in the mammalian adrenal gland (20, 21).

In conclusion, this observation shows that Cushing's syndromes associated with suppressed plasma ACTH levels may be dependent upon ACTH produced within the adrenocortical tissue. From a pathophysiological point of view, the term ACTH-independent used to designate primary adrenal Cushing's syndrome may therefore be inappropriate in some cases of bilateral adrenal macronodular hyperplasia with hypercorticism and undetectable plasma ACTH levels. However, the fact that plasma ACTH levels are suppressed in this unusual situation indicates that determination of plasma ACTH concentration remains the key diagnostic test for differentiating pituitary from adrenal causes of Cushing's syndrome.

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Address all correspondence and requests for reprints to: Dr. H. Lefebvre, European Institute for Peptide Research, Service d'Endocrinologie et Maladies Métaboliques, Hôpital de Boisguillaume, Centre Hospitalo-Universitaire de Rouen, 76031 Rouen Cedex, France. E-mail: herve.lefebvre@ chu-rouen.fr.

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