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Proliferation of the murine corticotropic tumour cell line AtT20 is affected by hypophysiotrophic hormones, growth factors and glucocorticoids

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Abstract

In pituitary-dependent hyperadrenocorticism (Cushing's disease), the disturbed regulation of ACTH secretion is associated with neoplastic transformation of corticotropic cells. As these two phenomena are almost indissolubly connected, it is of prime importance to elucidate the factor(s) that induce corticotropic cell proliferation. Here we report on the effects of hypophysiotrophic hormones and intrapituitary growth factors on the proliferation and hormone secretion of the murine corticotropic tumour cell line AtT20/D16v, as measured by DNA content, and ACTH concentration in culture media. In addition, sensitivity to the inhibitory effect of cortisol was assessed under various conditions. Corticotropin releasing hormone (CRH) and vasopressin (AVP) induced proliferation of AtT20-cells. In contrast to that caused by AVP, the CRH-induced proliferation was associated with increased ACTH secretion, which could be inhibited by cortisol. Insulin-like growth factor-I (IGF-I), epidermal growth factor (EGF) and basic fibroblast growth factor (bFGF) also stimulated the proliferation of AtT20-cells. The proliferation of AtT20-cells was significantly inhibited by cortisol in all tests. The IGF-I-induced proliferation was the least sensitive to inhibition by cortisol. The growth factors did not stimulate ACTH secretion but IGF-I differed in that it prevented the inhibition of basal ACTH secretion by cortisol. Additional experiments (Western ligand blot analysis) concerning the relative insensitivity of IGF-I induced proliferation to inhibition by cortisol revealed that IGF-I increased the concentration of a 29 kDa IGF binding protein (IGFBP) in the culture medium. The concentration of the 29 kDa IGFBP was slightly decreased by cortisol. In conclusion, the proliferation of AtT20-cells can be stimulated by the hypophysiotrophic hormones CRH and AVP and by the intrapituitary growth factors IGF-I, EGF and bFGF. Both basal and stimulated proliferation are sensitive to inhibition by cortisol, although this effect is remarkably low in the presence of IGF-I. IGF-I induced the secretion of a 29 kDa IGFBP, which might mediate the IGF-I effects by its intrinsic mitogenic properties. In addition to loss of sensitivity to endogenous glucocorticoids, high IGF-I concentrations may be a prerequisite for clonal expansion of pituitary corticotropes.

Keywords: AtT20-cell proliferation; Hypophysiotrophic hormone; Growth-factor; Cortisol; Insulin-like growth factor binding protein

1. Introduction

Most pituitary tumours are monoclonal in origin, as has been demonstrated by X-chromosomal inactivation analysis (Alexander et al., 1990; Herman et al., 1990; Schulte et al., 1991; Gicquel et al., 1992; Biller et al., 1992). This indicates that pituitary tumours arise from a single genomically altered cell. Subsequent clonal expansion is caused by either an intrinsic mutation resulting in a constitutive activation of cell replication or by extrinsic factors such as hypothalamic hormones or intrapituitary growth factors.

Examples of mutations occurring in pituitary tumours are the GTPase inhibiting mutations in the α-subunit of the GTP-binding protein (Gsα), which occur in about 40% of the growth hormone secreting adenomas (Vallar et al., 1987; Landis et al., 1990). Gsp mutations have recently also been found in 10% of non-functioning pituitary tumours (Tordjman et al., 1993) and in 5% of ACTH-secreting tumours (Williamson et al., 1993). A mutation in the H-ras proto-oncogen has been reported in

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one malignant prolactinoma (Karga et al., 1992) and DNA sequences from prolactinomas containing part of the coding region of the hst gene (a member of the FGF family) have been shown to transform NIH-3T3 cells (Gonsky et al., 1991). Invasive human pituitary tumours have been shown to express a point-mutated α-protein kinase-c (Alvaro et al., 1993).

However, such mutations have only been demonstrated in a minority of pituitary tumours (Herman et al., 1993; Cai et al., 1994) and the clonal expansion is still regarded as a major factor in the development of pituitary adenomas. It may require the action of hypothalamic hormones and/or intrapituitary growth factors. This notion is supported by the observations that GHRH transgenic mice develop somatotropic tumours (Asa et al., 1990) and CRH administration to rats increases the number of corticotropic cells (Gertz et al., 1987, McNicol et al., 1988). There is also evidence that GnRH has mitogenic properties for its pituitary target cells (Sakai et al., 1988).

The pituitary gland contains several growth factors (Webster et al., 1989; Halper et al., 1992), such as insulin-like growth factor-I (IGF-I) (Bach and Bondy, 1992), epidermal growth factor (EGF) (Kasselberg et al., 1985; Fisher and Lakshmanan, 1990), basic fibroblast growth factor (bFGF) (Baird et al., 1985; Ferrara et al., 1987; Gospodarowicz et al., 1987), IGF-II, transforming growth factor-α/β, and nerve growth factor (Webster et al., 1989; Halper et al., 1992). In the pituitary, localization of IGF-I, IGF-I receptor and IGFBPs (IGFBP-2, -3, -4, and -5) is not selectively related to the somatotroph population; therefore IGF-I may have effects on many pituitary cell types besides somatotrophs (Bach and Bondy, 1992). At least some of these factors may be involved in modulation of hormone secretion but much less is known about their effects on the replication of anterior pituitary cells (Webster et al., 1989).

The murine corticotropic tumour cell line AtT20 has been used extensively to study the regulation of ACTH release by corticotropic cells (Lundblad and Roberts, 1988). However, in these studies, effects on ACTH secretion have not been studied in connection with effects on the proliferation of corticotropic cells.

Here we report on the effects of corticotropic releasing hormone (CRH), arginine vasopressin (AVP), bFGF, EGF, and IGF-I on proliferation, ACTH secretion and synthesis, and secretion of IGFBPs of a murine corticotropic tumour cell line (AtT20). In addition, sensitivity to inhibition by cortisol was analysed under the various conditions.

2. Materials and methods

Unless indicated otherwise, chemicals were obtained from Sigma Co., St. Louis, MO, USA. The AtT20 cell line (subclone D16v) was generously supplied by Dr. J. Tooze (European Molecular Biology Organization; Heidelberg; Germany) and maintained in Eagle's minimum essential medium with Earle's salts supplemented with non-essential amino acids, L-glutamine (Biochrom KG, Berlin, Germany), 26.2 mM bicarbonate (Merck, Darmstadt, Germany), 10 mM Hepes (Seralab, Crawly Down, UK), 0.1 mM sodium pyruvate (Biochrom KG), 10% fetal calf serum (FCS) (Sebak, Aidenbach, Germany), 10 U/ml penicillin (Kombivet, Etten-Leur, The Netherlands) and 10 mg/ml streptomycin (Alfasan, Woerden, The Netherlands). Cells were grown at 37°C in a humidified atmosphere of 5% CO_2/95% air and subcultured after treatment with 0.25% (w/v) trypsin (Life Technologies, Breda, The Netherlands).

2.1. Proliferation studies

During the proliferation studies, the FCS in the medium described above was replaced by growth factor inactivated FCS (SH-FCS) and supplemented with 0.2% BSA, 30 nM Na_2SeO_3 and 10 μg/ml transferrin (Van Zoelen et al., 1985).

Cells were plated onto 24-well plates (Greiner, Alphen a/d Rijn, The Netherlands) at a density of 10^4 cells per well and grown for 2 days in medium with 10% FCS. Cells were washed and incubated with SH-FCS medium supplemented with 10 nM CRH (Peninsula Laboratories, St. Helens, Merseyside, UK), 100 nM AVP, 10 ng/ml bFGF, 10 ng/ml EGF, or 100 ng/ml IGF-I (bFGF, EGF, and IGF-I were obtained from Boehringer, Mannheim, Germany), with and without 100 nM cortisol. Doses were based on preliminary dose-response studies and maximal stimulating doses were chosen. After 4 days of incubation, the medium was changed. After 7 days, medium was removed from the cells. The cells were washed, lysed in 0.2% SDS, and stored at −20°C. DNA contents were measured using Hoechst 33258, based on the method described by Labarca and Paigen (1980). In short, buffer (10 mM EDTA, 10 mM Tris–HCl, 100 mM NaCl, pH 7.0) containing 100 ng/ml Hoechst 33258 and 5 μg/ml RNase (Boehringer) was added to the lysed cells and incubated for 15 min at room temperature. Fluorescence enhancement was measured and the DNA contents were read from the calibration curve constructed with calf thymus DNA (Boehringer).

2.2. Secretion studies

AtT20/D16v cells were plated onto 24-well plates at a density of 10^5 cells per well in medium with FCS, as described above. After 2 days, the cells were washed and incubated with medium in which FCS was replaced by 0.2% (w/v) BSA and supplemented with 10 nM CRH, 100 nM AVP, 10 ng/ml bFGF, 10 ng/ml EGF, or 100 ng/ml IGF-I, with and without 100 nM cortisol. After 4 h and 24 h, the medium was removed from the cells and stored at −20°C. The ACTH content of the medium was measured by RIA without extraction, according to the procedure of Arts et al. (1985). Antiserum was obtained

from IgG Corp. (Nashville, TN, USA). The tracer was purchased from International CIS (St. Quentin-Yvelines, France), and the standard was obtained from the NIH (Bethesda, MD, USA). The detection limit was 10 ng/l. Interassay variation was 12%.

2.3. Western ligand blot

AtT20/D16v-cells were grown in cell culture flasks (75 cm^2) (Greiner) until 80% confluency (10^7 cells). Then cells were washed and incubated with medium containing 0.2% (w/v) BSA and supplemented with 10 nM CRH, 100 nM AVP, 100 ng/ml IGF-I, 10 ng/ml EGF, or 10 ng/ml bFGF, with and without 100 nM cortisol. After 48 h the medium was removed from the cells and stored at −20°C. Twenty-microlitre samples of the conditioned media from the cell cultures were electrophoresed, electroblotted onto nitrocellulose membranes (Hybond C, Amersham, 's Hertogenbosch, The Netherlands), incubated with ^{125}I-labelled IGF-II (2×10^6 cpm/ 25 ml) overnight at 4°C, and exposed to X-ray film for 11 days. A low range prestained protein molecular weight marker was included.

2.4. RNA-isolation and northern blotting

AtT20/D16v-cells (4×10^7 cells) were grown in cell culture flasks (Greiner), washed and incubated with medium containing 0.2% (w/v) BSA and supplemented with 10 nM CRH, 100 nM AVP, 100 ng/ml IGF-I, 10 ng/ml EGF, or 10 ng/ml bFGF, with and without 100 nM cortisol. After 48 h, the cells were washed and RNA was isolated with the guanidine thiocyanate–phenol–chloroform method. Glyoxylated-RNA samples were electrophoresed through 1% agarose gels submerged in 10 mM sodium phosphate (pH 7.2) (Maniatis et al., 1982); transferred to a nylon membrane (Hybond N, Amersham, 's Hertogenbosch, The Netherlands), and hybridized with (1–2) × 10^6 cpm/ml of ^{32}P-labelled mIGFBP-1 to -6 cDNA probes (Schuller et al., 1994). Membranes were washed twice at 65°C in 0.1 M sodium phosphate (pH 7.2), 1% SDS, and 1 mM EDTA (pH 8.0) for 30 min. Membranes were exposed to X-ray film for 3 days.

2.5. Statistics

Eight proliferation studies (4–6 wells per incubation) and six secretion studies (4 wells per incubation) were conducted. Three of the secretion studies were performed with the addition of 100 nM cortisol to investigate the sensitivity to inhibition by cortisol. Results are expressed as percentage of the growth (proliferation studies) or ACTH secretion (secretion studies) in the control medium and presented as mean ± SEM.

Differences among the incubations were tested with the Kruskall–Wallis test, followed by the Mann–Whitney test, as corrected by Bonferroni. $P < 0.05$ was considered significant.

3. Results

3.1. Proliferation studies

The hypophysiotropic hormones CRH (157 ± 13%) and AVP (135 ± 6%) and the intrapituitary growth factors bFGF (228 ± 24%), EGF (251 ± 24%) and IGF-I (211 ± 13%) all significantly stimulated the proliferation of AtT20-cells in comparison to the proliferation in SH-FCS medium (100 ± 3%) (Fig. 1). The proliferation induced by CRH and AVP was significantly lower than that

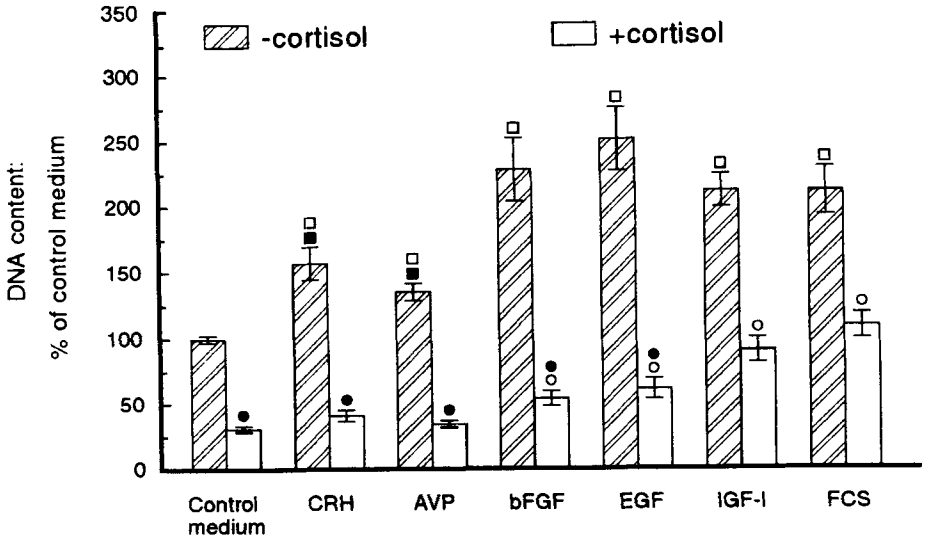

Fig. 1. Proliferation of AtT20/D16v-cells after 7 days of incubation with CRH (10 nM), AVP (100 nM), bFGF (10 ng/ml), EGF (10 ng/ml), or IGF-I (100 ng/ml) with (open bars) or without (hatched bars) 100 nM cortisol. Data are expressed as the percentage of growth in medium containing growth factor inactivated FCS (control medium) and presented as mean ± SEM. □, proliferation is significantly higher than in control medium; ■, proliferation is significantly lower than in medium with 10% FCS. O, proliferation is significantly higher than in control medium with cortisol; ●, proliferation is significantly lower than in medium with 10% FCS and cortisol. $P < 0.05$ was considered significant.

Table 1
Effects of CRH, AVP, bFGF, EGF, and IGF-I on ACTH secretion by AtT20/D16v-cells and the percentage change induced by cortisol after 4 and 24 h

	4 h incubation ACTH secretion (% of control)	% change with cortisol (100 nM)	24 h incubation ACTH secretion (% of control)	% change with cortisol (100 nM)
Control	100.0 ± 0.0	−6.8 ± 5.6	100.0 ± 0.0	−26.0 ± 2.6
CRH (10 nM)	179.7 ± 10.4*	−2.7 ± 7.6	147.2 ± 15.1*	−33.0 ± 3.7
AVP (100 nM)	93.6 ± 5.5	+12.5 ± 9.3	114.8 ± 9.5	−33.5 ± 2.2
bFGF (10 ng/ml)	107.8 ± 4.3	+17.5 ± 6.4	126.4 ± 8.8	−31.8 ± 6.3
EGF (10 ng/ml)	94.5 ± 3.9	−2.1 ± 5.6	113.0 ± 6.4	−33.3 ± 3.0
IGF-I (100 ng/ml)	117.1 ± 6.4	+14.8 ± 6.5	129.5 ± 6.6	−8.0 ± 4.6*

* $P < 0.05$.

induced by FCS (211 ± 18%), whereas the proliferation induced by the growth factors was not different from that induced by FCS.

The proliferation induced by the hormones CRH, AVP, and the growth factors bFGF, EGF and IGF-I was significantly inhibited by cortisol (Fig. 1). The basal proliferation of AtT20-cells was significantly inhibited by cortisol to 31 ± 2% of the proliferation in SH-FCS medium. Administration of CRH or AVP in combination with cortisol resulted in DNA contents of 41 ± 4% and 34 ± 3%, respectively, which were not significantly different from the proliferation in the SH-FCS medium with cortisol. Co-incubation of bFGF (54 ± 6%), EGF (61 ± 8%), or IGF-I (90 ± 10%) with cortisol resulted in significantly higher DNA contents than in SH-FCS medium with cortisol. In contrast to the other incubations with cortisol, the DNA contents after co-incubation of IGF-I with cortisol did not differ from the DNA contents in medium with FCS and cortisol.

3.2. Secretion studies

The ACTH secretion of AtT20-cells increased significantly after 4 h and 24 h of incubation with CRH, whereas AVP, bFGF, EGF, or IGF-I did not stimulate the ACTH secretion within 4 or 24 h (Table 1).

After 4 h of incubation, no significant inhibition of the

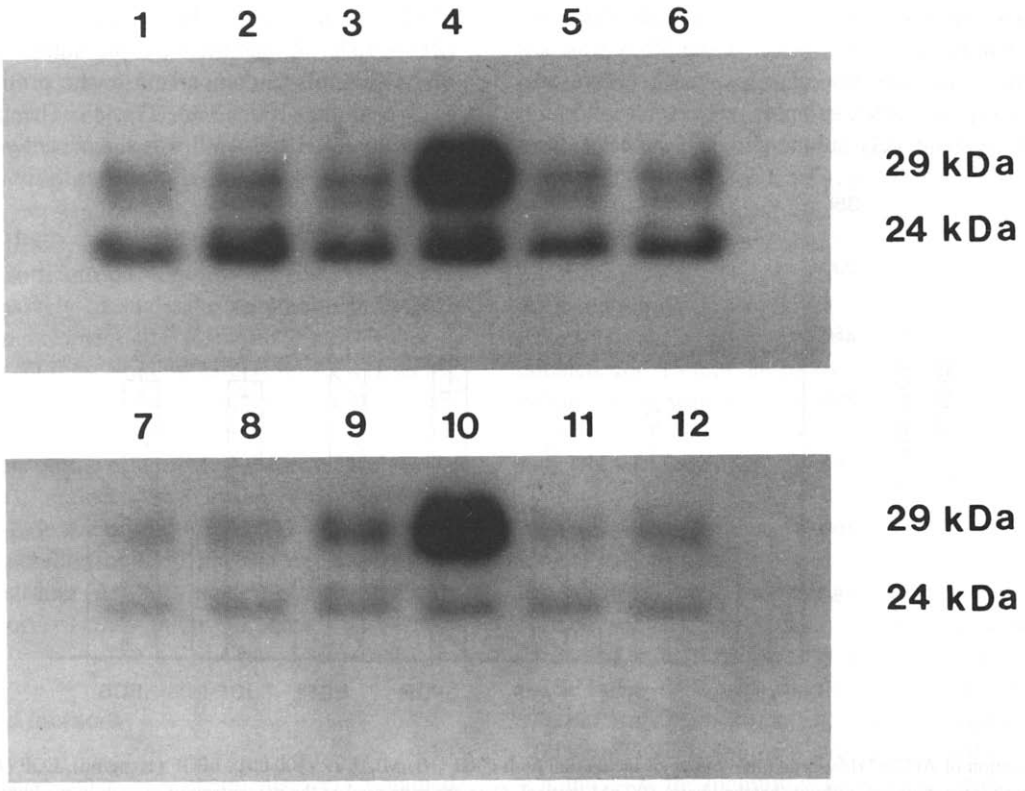

Fig. 2. Western ligand blot showing 24 kDa IGFBP and 29 kDa IGFBP in the conditioned media from AtT20/D16v-cell cultures. A representative western ligand blot is shown. Lanes: medium containing 0.2% (w/v) BSA (lanes 1, 7); 10 nM CRH (lanes 2, 8); 100 nM AVP (lanes 3, 9); 100 ng/ml IGF-I (lanes 4, 10); 10 ng/ml EGF (lanes 5, 11); 10 ng/ml bFGF (lanes 6, 12) incubated without (lanes 1–6) or with 100 nM cortisol (lanes 7–12).

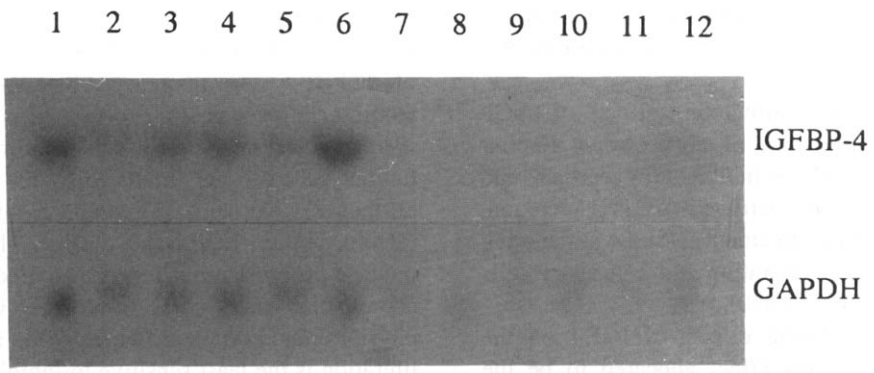

Fig. 3. Above: Northern blot showing IGFBP-4 (2.6 kb) expression in AtT20/D16v-cells after incubation with medium containing 0.2% (w/v) BSA (lanes 1, 7) supplemented with 10 nM CRH (lanes 2, 8), 100 nM AVP (lanes 3, 9), 100 ng/ml IGF-I (lanes 4, 10), 10 ng/ml EGF (lanes 5, 11) and 10 ng/ml bFGF (lanes 6, 12) incubated without (lanes 1–6) and with 100 nM cortisol (lanes 7–12). Below: Northern blot hybridization of the above blot with a GAPDH specific probe (0.9 kb).

ACTH secretion by cortisol was found. After 24 h of incubation, the ACTH secretion was significantly inhibited by cortisol in the control medium (26.0 ± 2.6%). In all other incubations the inhibition did not differ from that in the control medium, except for the incubation of IGF-I with cortisol. The inhibition by cortisol after co-incubation with IGF-I (8.0 ± 4.6%) was significantly lower than that in the control medium.

3.3. Western and northern blot analysis

The secretion of IGFBPs by AtT20/D16v-cells was studied by Western ligand blotting (Fig. 2). AtT20/D16v-cells secrete a 24 kDa IGFBP and a 29 kDa IGFBP (lane 1). Neither CRH, AVP, EGF, nor bFGF enhanced the secretion of the 24 kDa IGFBP or the 29 kDa IGFBP (lanes 2, 3, 5, 6). IGF-I stimulated the secretion of the 29 kDa IGFBP (lane 4). Cortisol inhibited the basal secretion of the 24 kDa IGFBP and the 29 kDa IGFBP (lane 7, 8, 9, 11, 12). A moderate inhibition of the IGF-I-stimulated 29 kDa IGFBP secretion was found after incubation with cortisol (lane 10).

Northern blot analysis revealed IGFBP-4 mRNA expression in incubations without cortisol (lane 1–6), which was inhibited by cortisol addition (lane 7–12). No mRNA expression of the other IGFBPs could be detected.

4. Discussion

In pituitary-dependent hyperadrenocorticism (Cushing's disease), the neoplastic transformation of corticotropic cells is associated with disturbed regulation characteristics, such as altered responsiveness to hypophysiotrophic hormones and decreased sensitivity to feedback by glucocorticoids. In studies on the pathogenesis of Cushing's disease, it is therefore important to know what factors may induce corticotropic cell proliferation and/or alter ACTH secretion. In the present study we investigated the effects of the hypophysiotrophic hormones CRH and AVP and the intrapituitary growth factors bFGF, EGF, and IGF-I on the proliferation and ACTH secretion of the murine corticotropic tumour cell line AtT20.

Our results confirm direct growth stimulating effects of CRH and AVP on corticotropic cells that have been observed in intact rats, in which, however, interactions of CRH and AVP with other factors or effects on cell populations other than corticotropes could not be excluded (Gertz et al., 1987; McNicol et al., 1988; McNicol et al., 1990). The CRH-induced proliferation was associated with increased ACTH secretion, whereas this was not true for the AVP-induced proliferation. This is compatible with the observation that AtT20 cells (D16-16) possess high affinity-low capacity receptors for AVP which are not coupled to secretion of POMC-derived peptides (Lutz-Bucher et al., 1987).

The CRH-induced ACTH secretion was not inhibited by cortisol after 4 h of incubation, whereas after 24 h of incubation a profound inhibition was found. In AtT20/D16-16 cells, CRH inactivates early glucocorticoid inhibition of CRH-stimulated ACTH secretion by rapidly inducing c-fos gene expression (Autelitano and Sheppard, 1993). This rapid elevation of fos results in the formation of the AP-1 complex (fos-jun complex) which binds to the glucocorticoid receptor and blocks its function (Yang-Yen et al., 1990). Our observation is in agreement with the results of Boutillier et al. (1991), who found that the CRH-mediated induction of the c-fos mRNA in corticotropic cells is transient (Boutillier et al., 1991).

However, according to current insights, the hypophysiotrophic hormones do not play an important role in the pathogenesis of Cushing's disease. In man and in the dog, it has been shown that the excessive ACTH secretion in Cushing's disease is not caused by persistent hyperstimulation of corticotropes by CRH (Kling et al., 1991; Van Wijk et al., 1992). In the present study, we found stimulating effects of CRH and AVP on the growth of corticotropic cells. This may, however, not be operational in pituitary-dependent hyperadrenocorticism, as we also

found that these effects were completely suppressed by glucocorticoids, which are intrinsically elevated in this disease.

In this study, we found stimulating effects of bFGF and EGF on the proliferation of corticotropic tumour cells. These effects could be inhibited by cortisol and were not associated with increased ACTH secretion. These observations are in agreement with the stimulating effects of bFGF on the proliferation of rat pituitary cells described by McNicol et al. (1990).

We also found stimulating effects of IGF-I on the growth of AtT20-cells. This effect appeared to be the least sensitive to inhibition by cortisol. The intrapituitary growth factors bFGF, EGF, and IGF-I induced proliferation of corticotropic cells and this effect was less sensitive to the feedback by glucocorticoids than the proliferation induced by hypophysiotropic hormones. Therefore, intrapituitary growth factors might stimulate the clonal expansion of corticotropic tumours.

Although we found that the growth factor-induced proliferation was not associated with increased ACTH secretion, IGF-I was able to prevent the glucocorticoid-provoked inhibition of basal ACTH release.

For further exploration of the relative insensitivity of IGF-I-induced proliferation to inhibition by cortisol, we examined the synthesis and secretion of IGF binding proteins (IGFBPs), because IGFBPs are known to modulate the effects of IGF-I. We found that AtT20-cells secrete a 24 kDa IGFBP and a 29 kDa IGFBP. Based on their molecular weights, these binding proteins represent IGFBP-4 and IGFBP-5, respectively. Neither CRH, AVP, bFGF, nor EGF enhanced the secretion of IGFBPs by AtT20-cells. The basal release of these IGFBPs was inhibited by the addition of cortisol. This confirms and extends the findings of Fielder et al. (1993), who found that AtT20-cells secrete IGFBP-4 and -5 and that IGF-I increases IGFBP-5 production at the protein and mRNA levels.

At the mRNA level we were able to demonstrate basal expression of IGFBP-4 which could be inhibited by cortisol. Although the stimulation of IGFBP-5 was convincingly demonstrated, we were not able to detect IGFBP-5 mRNA expression. In this study, cells were incubated for 48 h with cortisol, whereas in the study of Fielder et al. (1993) cells were incubated for 24 h with dexamethasone. In contrast to the study of Fielder et al. (1993), we did not observe a decrease in IGFBP-5 concentration following incubation with IGF-I and cortisol.

When all observations are considered, we have presented evidence that IGF-I plays a role in the clonal expansion of corticotropic adenomas, this especially because the IGF-I- induced stimulation of cellular division is less affected by cortisol than the proliferation induced by other intrapituitary growth factors. IGFBP-5 might mediate the actions of IGF-I because the IGF-I-induced secretion of IGFBP-5 is hardly sensitive to the inhibition by cortisol. Andress et al. (1993) found mitogenic effects of a truncated form of IGFBP-5 on osteoblasts. This form of IGFBP-5 also enhanced the IGF-I-stimulated osteoblasts mitogenesis.

In conclusion, the proliferation of murine corticotropic tumour cells can be stimulated by the hypophysiotrophic hormones CRH and AVP and the intrapituitary growth factors bFGF, EGF, and IGF-I. The growth factor-induced proliferation is less sensitive to inhibition by glucocorticoids than the proliferation induced by hypophysiotrophic hormones, whereby the IGF-I induced proliferation is the least sensitive to inhibition by glucocorticoids. Neither bFGF, EGF, nor IGF-I stimulate ACTH secretion by AtT20-cells but IGF-I impairs the inhibition of basal ACTH secretion by glucocorticoids. The IGF-I effects may be mediated by IGFBP-5. In ACTH-producing pituitary tumours, an increase in cell number is associated with a diminished sensitivity of ACTH secretion to inhibition by glucocorticoids. Not only loss of the sensitivity to endogenous glucocorticoids but also a high IGF-I concentration may be a prerequisite for clonal expansion of pituitary corticotropes.

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