Corticosteroids Stimulate the Differentiation of Growth Hormone Cells But Suppress That of Prolactin Cells in the Fetal Rat Pituitary

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Summary. An organ culture study was carried out to examine the effects of adrenal corticosteroids on the development of growth hormone (GH) cells and prolactin (PRL) cells. The adenohipophysial primordia were separated from 13.5-day-old fetal rats and maintained *in vitro* for 8 days with or without cortisol. Immunohistochemical examination of these explants showed that cortisol stimulated the differentiation of GH cells but suppressed that of PRL cells in a dose-dependent manner. In the absence of cortisol there were more PRL cells. Corticosterone had a similar effect on the developing adenohipophysis. When the pituitary primordia of Day 16.5 were cultured for 5 days and studied by the *in situ* hybridization technique, the expression of GH and PRL mRNA was found to be parallel with the immunoreactivity of the respective hormones. These data are discussed in relation to the normal development of GH and PRL cells in the fetal rat adenohipophysis.

The question of how different types of hormone-producing cells develop in the fetal pituitary has not been answered completely. Organ culture studies of undifferentiated adenohipophysial primordia of the rat have shown that, except for GH cells, hormone producing cells arise without inductive influence from other tissues (NEMESKÉRI and HALÁSZ, 1989). The question of what factor is involved in the differentiation of GH cells was answered by HEMMING et al. (1984, 1988), who demonstrated an indispensable role of cortisol in the development of GH cells. NOGAMI et al. (1995) confirmed this stimulative effect of glucocorticoids on differentiating GH cells.

At present, evidence suggests that there exists a close developmental relationship between GH and PRL cells. BEHRINGER et al. (1988) and BORRELLI et al.

(1989) found a marked decrease in the number of PRL cells in transgenic mice where GH cells were ablated by a genetic means. This fact led these investigators to suggest that GH and PRL cells are derived from a common precursor. SIMMONS et al. (1990) showed that both GH and PRL cells possess a protein referred to as Pit-1, which is known to activate GH and PRL promoters. It therefore seems of interest to investigate whether developing GH and PRL cells behave similarly in response to glucocorticoid treatment. The administration of corticosteroids was reported to have a suppressive effect on PRL gene expression in the adult rat pituitary (ADLER et al., 1988), but to date the effect of corticosteroids on developing PRL cells has received no attention. In view of these findings, an *in vitro* experiment was undertaken where the pituitary primordia of fetal rats were cultured in the presence of adrenocortical steroids.

This paper demonstrates that glucocorticoids have suppressive influences on differentiating PRL cells in contrast to the stimulative role of these steroids in the development of GH cells.

MATERIALS AND METHODS

Animals and organ culture

Adult rats of the Sprague-Dawley strain were mated at night. If spermatozoa were found in vaginal smears the next morning, that day was designated as Day 0.5 of gestation. Pregnant rats at Days 13.5 and 16.5 of gestation were anesthetized with ketamine hydrochloride and fetuses were removed for microsurgery. In this study, pituitary primordia were cultured with the surrounding brain and mesenchymal

tissues. The organ culture was performed as described previously (WATANABE, 1987). The primordia explanted on Day 13.5 were kept for 8 days. In view of the data that cortisol is the main adrenal steroid in the rat fetus (KALAVSKY, 1971), we first tested the effect of cortisol and then that of corticosterone. Both steroids were purchased from Sigma (MO, USA) and added to the medium at concentrations of 50, 275 and 500 nM. Corticosterone was first solved in ethanol and diluted to the final concentrations with distilled water. For controls, vehicles were added. At the end of the culture period, explants were fixed in Bouin's solution. After removing cellulose acetate membranes in ethanol, they were embedded in paraffin and cut at 2 μ m for immunohistochemistry. For *in situ* hybridization, the pituitary primordia were separated on Day 16.5 because the primordia explanted on Day 13.5 were too small to be processed for frozen sectioning. These cultures were maintained for 5 days.

Immunohistochemistry

After deparaffinization, sections were immunohistochemically stained according to the PAP method by the use of anti-rat GH (HAC-RT25-01-RBP85) and anti-rat PRL (HAC-RT26-01-RBP85) which were kindly supplied by Dr. K. WAKABAYASHI (Gunma University, Japan). For cell counting, every 7th section was mounted on the same slide, and immunostained for both the antisera. The number of immunoreactive cells on each slide was counted.

In situ hybridization

Culture explants were fixed in 4% paraformaldehyde in 0.1 M phosphate buffer for 24 h at 4°C. The fixed explants were immersed in 30% sucrose in PBS at 4°C overnight, embedded in OCT, and stored at -80°C. Cryostat sections were cut at 5 μ m and mounted on APS-coated slides (Matsunami, Japan).

Rat GH cDNA was kindly supplied by Dr. J. D. BAXTER (University of California, San Francisco, CA), and rat PRL cDNA was a gift from Dr. A MAUER (University of Iowa, IA). All reagents used for *in situ* hybridization were purchased from Boehringer Mannheim, Germany. *In vitro* transcription was performed with the DIG RNA labeling kit according to the manufacturer's protocol. Digoxigenin-labelled RNA probes were precipitated with ethanol and dissolved in 50 ml of dimethyl-dicarbonate-treated water. The probes were diluted (1: 100) with a hybridization solution consisting of 2% of a blocking reagent, 5 $\times$ SSC, 50% formamide, and then denatured for 2 min at 90°C and chilled in ice. The slides were incubated with a hybridization solution

containing denatured RNA probes at 45°C for 16 h. After three washes with buffer A (100 mM Tris-HCl, pH 7.5, 150 mM NaCl), the sections were treated with a 0.5% blocking reagent in buffer A for 30 min at room temperature followed by incubation with anti-digoxigenin-AP, Fab fragments (1: 500) in buffer A for 1 h. The sections were then washed in buffer A three times and then in buffer B (100 mM Tris-HCl, pH 9.5, 100 mM NaCl, 50 mM MgCl₂). Finally, the sections were incubated with 4-nitro blue tetrazolium chloride (33.5 μ g/ml) and 5-bromo-4-chloro-3-indolyl-phosphate (165 μ g/ml) in buffer B at room temperature in darkness.

RESULTS

Effect of cortisol on the number of immunoreactive GH and PRL cells

When the pituitary primordia of 13.5-day-old rat fetuses were removed and cultured for 8 days without cortisol, no GH cells were observed (Fig. 1a). In the presence of cortisol, on the other hand, all culture explants were found to contain GH cells (Fig. 1b-d). In cortisol-treated explants, GH cells first appeared on the 4th day of culture. The numbers of immunoreactive cells increased through the duration of the organ culture. Figure 2 summarizes the results of cell counting. The numbers of GH cells increased in parallel with the cortisol concentrations.

Unlike GH cells, PRL cells were most frequently found when the pituitary primordia were cultured without cortisol (Fig. 1e). Thus, at a concentration of 500 nM cortisol, only a few or no PRL cells were observed (Figs. 1h, 2).

When cortisol was replaced with corticosterone, almost identical results were obtained (data not shown).

Effect of cortisol on GH and PRL mRNA expression

As already described, the pituitary primordia for *in situ* hybridization were separated on Day 16.5 of fetal age and cultured with or without cortisol (275 nM) for 5 days. As expected, GH and PRL transcripts were found only when culture explants possessed immunoreactive cells in both cases. Thus no GH transcripts were observed in those explants kept without cortisol (Fig. 3a). In the presence of cortisol many cells were found to express GH transcripts (Fig. 3b). Conversely, PRL transcripts were abundantly detected in the absence of cortisol (Fig. 3c), whereas little PRL mRNA was present when explants were maintained with cortisol (Fig. 3d).

Fig. 1. Portions of fetal rat pituitary explanted on Day 13.5 and grown in α MEM containing 0 nM (a and e), 50 nM (b and f), 275 nM (c and g) and 500 nM (d and h) of cortisol for 8 days. Co-cultured brain (*asterisk*) and sphenoid cartilage (s) are also seen. Sections immunohistochemically stained for GH (a-d) and PRL (e-h). $\times 115$

Fig. 2. The numbers of immunoreactive GH (a) and PRL (b) cells contained in the fetal rat pituitary primordia explanted on Day 13.5 and kept with varying doses of cortisol for 8 days. Note dose-dependent stimulative and suppressive effects of cortisol on developing GH and PRL cells, respectively.

Fig. 3. Frozen sections of fetal rat pituitary explanted on Day 16.5 and grown in α MEM with 0 nM (a, c) and 275 nM (b, d) of cortisol. Sections were processed for *in situ* hybridization with digoxigenin-labeled GH cDNA (a, b) or PRL cDNA (c, d). $\times 230$

DISCUSSION

Adenohypophysial hormone-producing cells do not differentiate at the same time during development. In fetal rats, the ACTH cell is the first cell type known to appear as early as Day 15 of gestation (DUPOUY and DUBOIS, 1975; SÉTÁLO and NAKANE, 1976; WATANABE and DAIKOKU, 1979; DUPOUY, 1980; CHATELAIN and CHEONG, 1986; NEMESKÉRI et al., 1988). During the subsequent 2–3 days, the TSH cells and gonadotropes come to be immunohistochemically detectable. Finally, on Days 18–19, GH and PRL cells appear almost simultaneously (NOGAMI et al., 1989; WATANABE and HARAGUCHI, 1994). The interpretation of such sequential differentiations of various cell types was previously difficult. We can, however, now propose an explanation as to why GH cells differentiate considerably later than ACTH cells. In the first place, it should be emphasized that the fetal adenohypophysis is indispensable for the development of the fetal adrenal gland (KITCHELL, 1950; KITCHELL and WELLS, 1952; COHEN, 1960; JOST, 1966; KALAVSKY, 1971). Soon after their differentiation in the fetal hypophysis, ACTH cells undergo rapid proliferation (WATANABE and DAIKOKU, 1979). Evidence indicates that plasma ACTH can be detected by radioimmunoassay in 17-day-old rat fetuses, with a peak on Day 19 (CHATELAIN and CHEONG, 1986). Such an elevated level of ACTH is responsible for the release of corticosteroids, which have actually been detected in the serum of fetal rats from Day 17 (COHEN, 1973) or Day 18 (KALAVSKY, 1971). It must be emphasized that maternal adrenalectomy has little influence upon the serum corticosterone concentration of fetuses (DUPOUY et al., 1975). Under the influence of circulating corticosteroids mostly of fetal origin, the differentiation of GH cells takes place as demonstrated in the present study and in other *in vivo* (NOGAMI et al., 1989) and *in vitro* experiments (HEMMING et al., 1984). Thus the synthesis and release of ACTH from the fetal pituitary must occur before the differentiation of GH cells takes place.

Although corticosterone is known to be the major corticosteroid in rats as in other rodents, there is a notable difference between the adult and fetus. KALAVSKY (1971) showed that the adrenal cortex of 18-day-old fetal rats contained far greater amounts of cortisol than of corticosterone. This finding is supported by the *in situ* hybridization study by KEENEY et al. (1995), who have demonstrated that the adrenal cortex of the fetal mouse transiently expresses mRNA of 17 α -hydroxylase cytochrome P450 that is

required for the production of cortisol. Our study showed that both cortisol and corticosterone were found to induce the differentiation of GH cells. Cortisol, however, is more likely involved in the actual cytogenetical process of GH cells in view of the production of this steroid in correspondence with the stage (Day 18) when GH cells differentiate in the fetal rat pituitary.

In the present study, the differentiation of PRL cells was suppressed in the presence of corticosteroids. As already described, blood levels of glucocorticoids in fetal rats are high at Day 18 when the first GH and PRL cells appear in the pituitary. This fact well explains why the number of PRL cells remains very low while GH cells undergo a marked increase in number between Days 18 and 21 (WATANABE and HARAGUCHI, 1994). A quantitative Western blot analysis performed by NAGATA et al. (1992) showed that the pituitary content of PRL increases only slightly from Days 19 to 21, being twofold versus tenfold for that of GH. According to the present data, the number of immunoreactive PRL cells was greatest in the absence of glucocorticoids. Thus the differentiation of PRL cells may be a 'default' pathway in the fetal adenohypophysis. Why the development of GH cells has priority over that of PRL under the influence of circulating glucocorticoids remains to be determined.

Evidence presently available indicates that a pituitary specific transcription factor called Pit-1 (GHF-1) is involved in the differentiation of both GH and PRL cells. Although no effort was made to detect this transcription factor in the present study, glucocorticoid probably suppressed Pit-1 expression, at least in PRL cells. This postulation comes from the observation that Pit-1 expression in PRL cells is inhibited by glucocorticoids (SUBRAMANIAM et al., 1997). As for GH cells, on the other hand, NOGAMI et al. (1997) have provided data suggesting that the induction of GH mRNA by glucocorticoids in the fetal pituitary is not through a direct effect by these steroids, but rather is mediated by a newly synthesized protein. Further studies are needed for determining the effect of corticosteroids on GH expression in the developing rat pituitary.

On the basis of experiments in which GH-expressing cells were genetically deleted, most investigators are inclined to think that not only GH cells but also PRL cells are derived from a common GH-producing stem cell. In the present study, culture explants contained only PRL cells in the absence of corticosteroids. This raises the question as to whether these PRL cells once produced GH during an early stage of the culture period. We could not detect any GH cells by immunohistochemistry, at least in the explants

that were removed at Day 13.5 and maintained for 5–8 days in culture (data not shown). However, in considering the small amounts of pituitary hormones—particularly in the initial phase of development, further cytological studies are necessary for determining how PRL cells differentiate in the absence of corticosteroids.

Recent immunocytochemical investigations have shown the presence of bihormonal cells containing both GH and PRL at the same time in the adenohypophysis of rats (BOOCKFOR et al., 1986; NIKITOVITCH-WINTER et al., 1987; PORTER et al., 1990, 1991; NOGAMI et al., 1995), cows (HASHIMOTO et al., 1985) and mice (SASAKI and IWAMA, 1989). The occurrence of such bihormonal cells, or somatomammotropes, also suggests that there exists a close developmental relationship between GH and PRL cells. However, according to our previous immunohistochemical data on the fetal pituitary (WATANABE and HARAGUCHI, 1994), the development GH and PRL cells may be independent. We observed that the GH cells differentiate predominantly in the posterior half of the fetal rat pituitary gland, whereas PRL cells arise in its anterior part. This finding suggests the possibility that the segregation of precursors of GH and PRL cells already occurs at an early stage of development. If this is the case, it is conceivable that the glucocorticoids influence each of the two types of precursor cells separately. Further work is needed to clarify the relationship between developing GH and PRL cells during fetal life.

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