

# Multistep differentiation of GH-producing cells from their immature cells

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## Abstract

In order to study GH cell differentiation, we used the clonal cell lines called MtT/E and MtT/S cells, which were derived from a rat mammothrophic pituitary tumor. Although MtT/E cells are non-hormone-producing ones, Pit-1 protein is present in their nuclei, which suggests that MtT/E cells are progenitor cells of the Pit-1 cell lineage and have the potential to differentiate into hormone-producing cells. On the other hand, MtT/S cells produce GH; however, the responsiveness to GH-releasing hormone (GHRH) is weak and only a small number of secretory granules are present in their cytoplasm, which suggests that MtT/S cells are premature GH cells. In order to differentiate into GH cells from MtT/E cells as a progenitor cell, we examined several differentiation factors and found that retinoic acid (RA) induced the differen-

tiation of MtT/E cells into GH-producing cells. RA-induced GH cells partially matured with the glucocorticoid treatment; however, the responsiveness to GHRH on GH secretion was incomplete. In order to elucidate the mechanism underlying full differentiation of GH cells, we used MtT/S cells. We treated MtT/S cells with glucocorticoid and found that they differentiated into mature GH cells with many secretory granules in their cytoplasm and they responded well to GHRH. These results suggested that MtT/E and MtT/S cells are progenitor or premature GH cells, and show different responses to differentiation factors. Our data also suggested that GH cells differentiate from their progenitor cells through multistep processes.

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## Introduction

The endocrine cells in the anterior pituitary gland can be classified into five phenotypes according to their different hormone production, i.e. somatotroph, lactotroph, thyrotroph, gonadotroph and corticotroph. These cells are considered to arise from a common precursor cell, and the sequential expression of transcription factors involved in pituitary organogenesis induces differentiation into different endocrine cells. Pituitary endocrine cell development and the specific production of these transcription factors may be controlled by some extracellular signaling molecules, such as fibroblast growth factor 8, bone morphogenetic protein 4 and Wnt5a (Treier *et al.* 1988, Sheng & Westphal 1999, Dasen & Rosenfeld 2001), and steroid hormones (Nogami *et al.* 1995, Stahl *et al.* 1999, Matsubara *et al.* 2001). In addition, the potent ability of cytokines such as leukemia inhibitory factor (Akita *et al.* 1997) might

be considered to be essential for pituitary development. However, the mechanism and process of differentiation of pituitary endocrine cells are still under discussion. The endocrine stem cells or progenitor cells in the embryonic or adult pituitary gland have not been well studied.

On the other hand, Pit-1/growth hormone factor-1 has been found to be a transacting factor for growth hormone (GH) and prolactin (PRL) (Bodner *et al.* 1988, Ingraham *et al.* 1988, Nelson *et al.* 1988), and is known to be essential for the differentiation and survival of these cells. In fact, Snell and Jackson dwarf mice are known to have a point mutation of the Pit-1 gene, and are also characterized by deficiency in somatotroph, lactotroph and thyrotroph (Li *et al.* 1990). These results indicate that these endocrine cells may differentiate from their common Pit-1-positive progenitor cells. In addition, it is reported that synthesis of Pit-1 protein precedes GH mRNA expression in fetal pituitary gland which suggests that Pit-1

progenitor cells exist in the developing pituitary gland (Simmons *et al.* 1990). The effect of glucocorticoid and thyroid hormone on GH cell development has also been reported (Nogami & Tachibana 1993, Rodriguez-Garcia *et al.* 1995). However, the induction of GH cells from pit-1 progenitor cells *in vitro* has not been performed.

In this study, we used two cell lines, called MtT/S and MtT/E, derived from an estrogen-induced mammothrophic tumor (MtT/F84) (Inoue *et al.* 1990). These cell lines are characterized by their structure, hormone production and response to estrogen on cell growth. MtT/E cells do not produce any pituitary hormones and their growth is not stimulated by estrogen (Inoue *et al.* 1990). The effect of interleukin-6 (IL-6) on cell growth is also different from the other endocrine cell lines (Sawada *et al.* 1995). Interestingly, MtT/E cells are positive for Pit-1, which strongly suggests that MtT/E cells are committed to an endocrine cell lineage. We have therefore hypothesized that MtT/E cells are progenitor cells of the pituitary endocrine cells and have the potential to differentiate into Pit-1-positive endocrine cells.

In contrast, MtT/S cells are GH-producing ones and their character as endocrine cells has been well studied (Goda *et al.* 1998, Nogami *et al.* 2000). MtT/S cells produce GH in normal medium; however, the responsiveness to GH-releasing hormone (GHRH) is weak and only a few secretory granules are present in their cytoplasm, which suggests that MtT/S cells are premature GH ones. In this paper, we report that retinoic acid (RA) induces the differentiation of GH cells from non-hormone-producing cells, and the glucocorticoid causes the maturation of GH cells.

## Materials and Methods

### Cell culture

MtT/E and MtT/S cells were established from an estrogen-induced mammothrophic tumor, MtT/F84 (Inoue *et al.* 1990). The cells were cultured in a 1:1 mixture of Ham's F12 medium (Life Technologies, Inc., Grand Island, NY, USA) and Dulbecco's minimum essential medium (Life Technologies). The medium was supplemented with 0.45% glucose, 55 mg/l pyruvic acid, 2.85 g/l sodium bicarbonate, 100 mg/l kanamycin (Meiji Seika Ltd, Tokyo, Japan), 15.5 mg/l penicillin (Meiji Seika Ltd), 25 mg/l streptomycin (Meiji Seika Ltd), 10% normal horse serum (NHS; Nichimen America, Los Angeles, CA, USA) and 2.5% fetal bovine serum (FBS; Biowhittaker Inc., Walkersville, MD, USA). The cells were cultured in a CO<sub>2</sub> incubator at 37 °C under a humidified atmosphere of 5% CO<sub>2</sub> in air.

### Chemicals and growth factors

The following chemicals were used in this study: vasoactive intestinal peptide (VIP; Peptide Institute, Osaka,

Japan), pituitary adenylate cyclase-activating polypeptide (PACAP-38; Peptide Institute), GH-releasing peptide (Peptide Institute), *all-trans*-RA (Sigma Co., St Louis, MO, USA) and corticosterone (Sigma Co.). Recombinant human basic fibroblast growth factor (bFGF) was a generous gift from Takeda Chemical Industries (Osaka, Japan).

### Immunocytochemistry

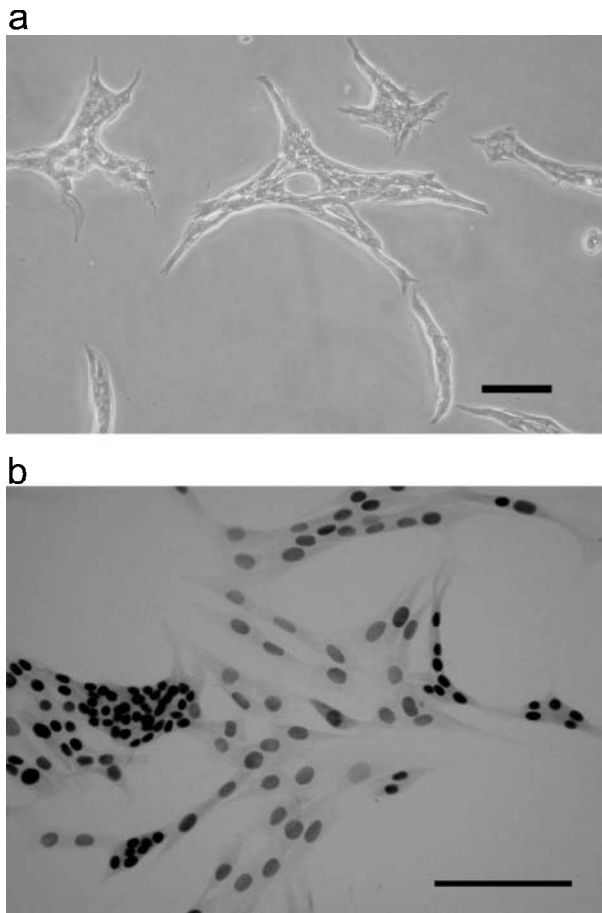
The cells on 35 mm culture dishes were fixed with 10% formalin in phosphate-buffered saline (PBS) for 1 h, and then preincubated in PBS containing 1% NHS and 0.4% Triton X-100 (TNBS). The cells were then incubated with rabbit anti-rat GH antibodies (HAC-RT25-02 RBP85; a gift from Dr K Wakabayashi) diluted 1:2000 or antibodies to Pit-1 (a gift from Dr M G Rosenfeld) diluted 1:1000 with TNBS overnight, followed by goat anti-rabbit IgG (HAC-RBA2-04 GTP86; a gift from Dr K Wakabayashi) diluted 1:5000 with TNBS for 2 h. After rinsing with PBS, the cells were reacted with a peroxidase-anti-peroxidase immunocomplex (PAP-complex; Dako, Copenhagen, Denmark) diluted 1:200 with TNBS for 1 h. The immunoreactive cells were visualized with 3,3'-diaminobenzidine. For confocal laser microscopical observation, MtT/S cells were reacted with anti-rat GH, as noted above, and then further incubated with fluorescein isothiocyanate-conjugated anti-rabbit IgG (Cappel, Cochranville, PA, USA). Samples were observed under a confocal laser microscope (Carl Zeiss Inc., Oberkochen, Germany).

### Measurement of medium IL-6 levels

To study the effects of PACAP and VIP on IL-6 secretion, cells were loaded into 24-well plates at  $5 \times 10^4$  cells/well. Two days later, the medium was changed to fresh medium containing different concentrations of PACAP-38 or VIP. After 24 h, the conditioned media were used for IL-6 measurement. The IL-6 concentrations in the media were determined with a bioassay system, using an IL-6 sensitive mouse hybridoma cell line (B9) (supplied by Dr L A Arden, Netherlands Red Cross Blood Transfusion Center, Amsterdam, The Netherlands). The B9 cells were cultured in RPMI 1640 medium supplemented with 10% FBS. Recombinant IL-6 (2 µl/well) or conditioned medium (2 µl/well) were added to the culture medium of B9 cells (198 µl/well), followed by further culture for 72 h. After cultivation, the growth of B9 cells was measured using fluorescent Alamar Blue (Alamar Bio-Sciences Inc., Sacramento, CA, USA). The assay limit of this system is 1 pg.

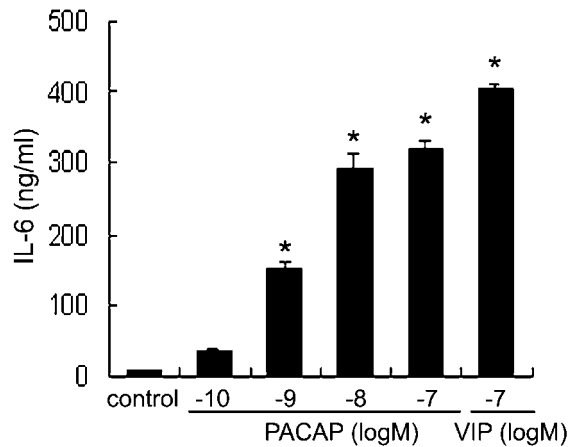
### Electron microscopy

MtT/S cells were incubated with or without  $1 \times 10^{-8}$  M corticosterone for 24 h in a culture flask. MtT/E cells



**Figure 1** The morphological characters of MtT/E cells. (a) Light microscopic appearance of MtT/E cells. MtT/E cells are spindle shaped. Scale bar=10 µm. (b) Immunocytochemical localization of Pit-1 in MtT/E cells. Pit-1 was detected in the nuclei of MtT/E cells. Scale bar=10 µm.

stimulated for 5 days with RA ( $1 \times 10^{-6}$  M) were incubated with or without corticosterone ( $1 \times 10^{-8}$  M) for 24 h. After removal of the medium, the cultured cells were prefixed with 2.5% glutaraldehyde for 30 min and then post-fixed with 1% osmium tetroxide for 30 min. The cells in the culture flasks were harvested with a cell scraper and centrifuged. The precipitated cell pellets were then resuspended in horse serum and centrifuged again. After removal of the supernatant, the cell pellets were fixed again with 2.5% glutaraldehyde for 1 h. Through this post-fixation, the pellets in the serum become stable and easy to handle in the following dehydration. After dehydration with an ascending ethanol series, the pellets were collected from the centrifuge tube and embedded in epoxy resin according to a routine method. Ultrathin sections were stained with uranyl acetate and lead citrate, and then examined under a transmission electron microscope (H-7500; Hitachi High-Technologies Corp., Hitachi, Japan).



**Figure 2** IL-6 secretion from MtT/E cells. The IL-6 secretion was stimulated by PACAP in a dose-dependent manner, and VIP also stimulated IL-6 secretion from MtT/E cells. MtT/E cells were loaded onto 24-well plates. Two days later, the cells were stimulated with PACAP or VIP for 24 h, and then the conditioned medium was collected and assayed for the IL-6 concentration. Values are means  $\pm$  s.e. ( $n=4$ ). \* $P<0.001$  vs control.

#### Radioimmunoassay

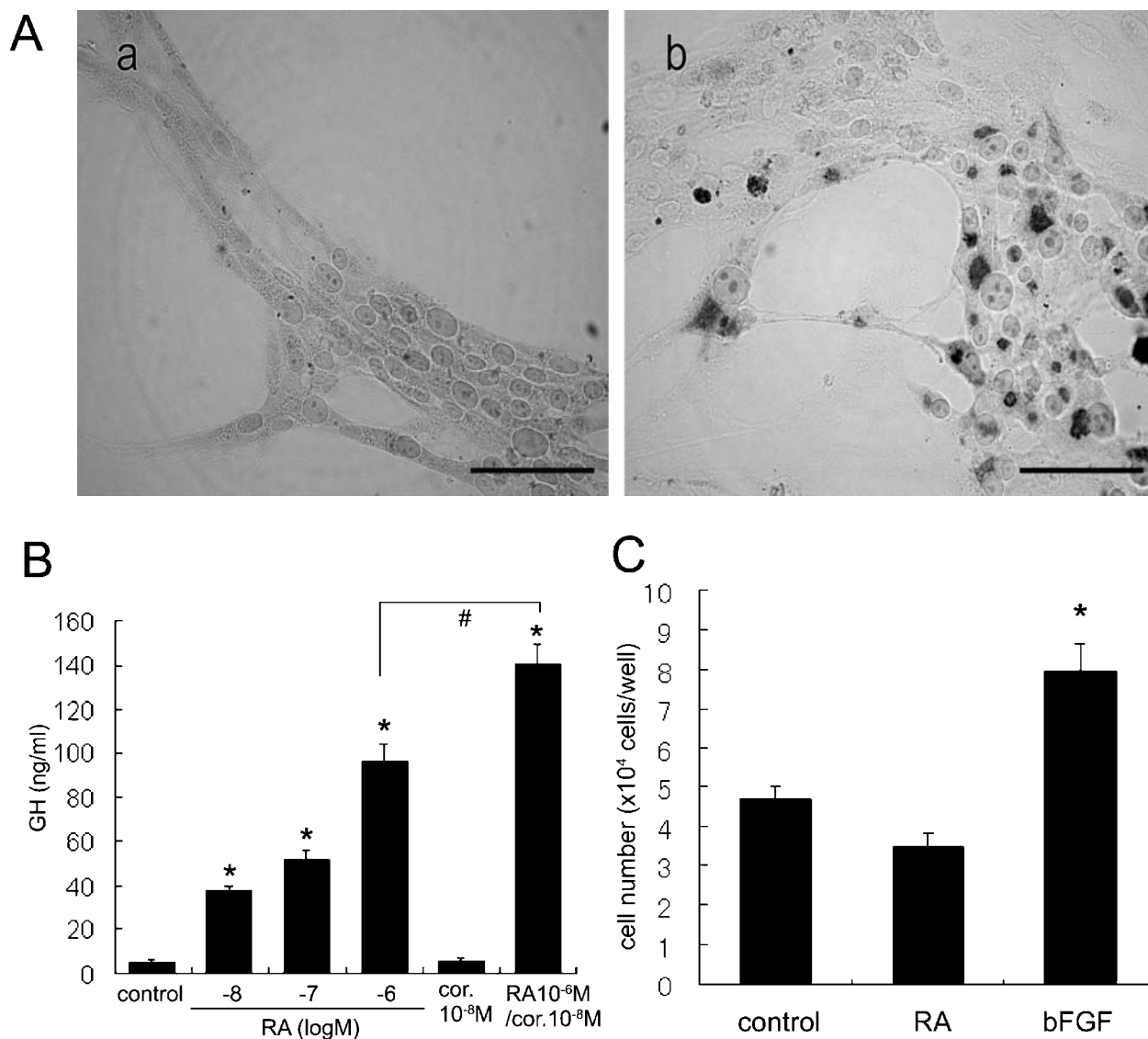
Double-antibody RIAs were performed for determination of medium and cellular GH levels. For determination of the cellular GH levels, cells were extracted with PBS containing 2 M urea (pH 7.5). Standard hormones (GH: RP-2) and antibodies (GH: S-4) for RIAs were supplied by the National Hormone and Peptide Program (Bethesda, MD, USA).

#### Effects of bFGF, RA and corticosterone on cell growth

In order to examine cell growth, MtT/E cells were seeded into 24-well plates at  $2 \times 10^4$  cells/well using charcoal dextran (Sigma Co.)-treated medium. Two days later, the culture medium was changed to fresh medium with or without estrogen, bFGF and RA. Moreover, 4 days later, viable cells were dispersed with 0.025% trypsin and then counted with a hemocytometer. MtT/S cells were seeded at  $1 \times 10^4$  cells/well, and after 4 days stimulation with corticosterone ( $1 \times 10^{-10}$  to  $1 \times 10^{-6}$  M) they were counted with a hemocytometer.

#### Statistical analysis

The data are expressed as the means  $\pm$  s.e. for independent experiments repeated at least three times. Statistical analysis was performed using Fisher's protected least significant difference test, differences at  $P<0.05$  being considered significant.



**Figure 3** Effect of RA on the differentiation of GH-producing cells from non-hormone-producing cells among MtT/E cells. (A) Immunocytochemical detection of GH in MtT/E cells. Note that no GH-immunopositive cells were observed in (a) the control, but (b) many GH-positive cells appeared on treatment with  $10^{-6}$  M RA for 6 days. Scale bars = 100  $\mu$ m. (B) Effect of RA on GH secretion from MtT/E cells. MtT/E cells were incubated with RA ( $1 \times 10^{-8}$  M to  $1 \times 10^{-6}$  M) and/or corticosterone (cor.;  $1 \times 10^{-8}$  M) for 6 days. The GH secretion from MtT/E cells was stimulated by RA in a dose-dependent manner. Only corticosterone treatment for 6 days had no effect on GH secretion. Values are means  $\pm$  S.E. ( $n=3$ ). \*,  $P<0.0001$  vs control; #,  $P<0.0001$  vs RA  $10^{-6}$  M. (C) Effects of bFGF and RA on the growth of MtT/E cells. The proliferation of MtT/E cells was stimulated by bFGF, but suppressed by RA. The stimulation by bFGF and RA was demonstrated for 4 days using charcoal dextran-treated medium, and the cell number was determined with a hemocytometer. Values are means  $\pm$  S.E. ( $n=4$ ). \* $P<0.001$  vs control.

## Results

### Morphological appearance of MtT/E cells, and effects of PACAP and VIP on IL-6 secretion

The light microscopic appearance of MtT/E cells is shown in Fig. 1a. The cells had a flat appearance and adhered well to the culture dishes. Spindle-shaped cells were also

observed. Immunocytochemistry showed that they were not positive for any antibodies to anterior pituitary hormones (data not shown); however, they were stained with antibodies for Pit-1 protein, and their nuclei were stained densely, as shown in Fig. 1b. The IL-6 secretion of MtT/E cells was stimulated by PACAP and VIP, as shown in Fig. 2.

### Effects of RA, glucocorticoid and bFGF on GH cell differentiation from MtT/E cells

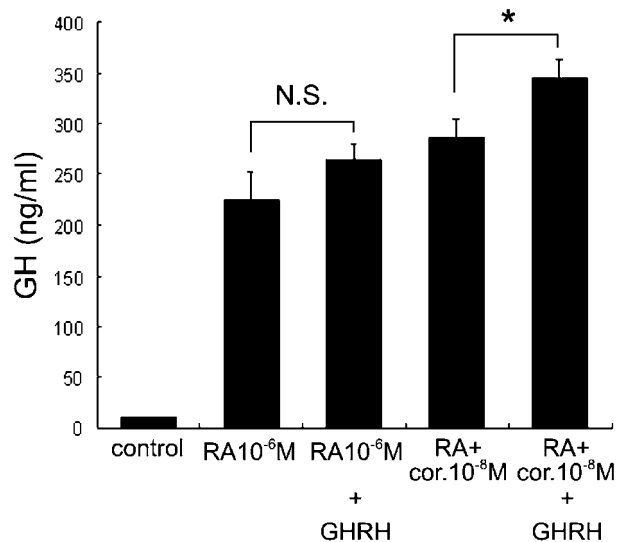
In order to obtain GH cells from MtT/E cells, we stimulated MtT/E cells with RA and found that GH-immunopositive cells appeared after treatment with RA. As shown in Fig. 3A(b), many GH-producing cells, which can be distinguished by the specific staining of the Golgi apparatus, appeared among the MtT/E cells on treatment with RA ( $1 \times 10^{-6}$  M) for 6 days. In contrast no GH-positive cells were seen in the control (Fig. 3A(a)). Both the medium GH level and the number of GH-immunopositive cells were increased with the RA treatment. As shown in Fig. 3B, corticosterone ( $1 \times 10^{-8}$  M) itself did not increase the GH production by MtT/E cells in the media; however, when corticosterone was used with RA, the medium GH level with MtT/E cells increased additively. The RA-induced GH-immunopositive cells did not disappear on further cultivation in the medium without RA (data not shown), which suggests that GH-immunopositive cells do not exhibit transient production of GH. We next examined the effects of RA and bFGF on the growth of MtT/E cells. As shown in Fig. 3C, the growth of MtT/E cells was stimulated by bFGF but was inhibited slightly by RA.

For the full differentiation of GH cells from MtT/E cells, we preincubated MtT/E cells with RA for 5 days then additionally stimulated with or without corticosterone ( $1 \times 10^{-8}$  M) for 24 h, and then stimulated with GHRH ( $1 \times 10^{-8}$  M) or the vehicle for 30 min. As shown in Fig. 4, MtT/E cells stimulated with RA only did not respond to GHRH; however, the cells stimulated with both RA and corticosterone responded to GHRH slightly. Electron microscopic observation showed that GH cells stimulated with RA only had no secretory granules in their cytoplasm, as shown in Fig. 5a. In contrast, MtT/E cells stimulated with RA and glucocorticoid had a small number of tiny secretory granules in their cytoplasm, specifically around the Golgi apparatus (Fig. 5b).

### Effect of glucocorticoid on the differentiation of MtT/S cells

Almost all MtT/S cells are considered to be GH-producing ones, but GH immunoreactivity is restricted to the Golgi apparatus, and they do not respond to GHRH. In order to determine the effect of corticosterone on GH cells, MtT/S cells were incubated with corticosterone (0,  $1 \times 10^{-10}$  M,  $1 \times 10^{-8}$  M and  $1 \times 10^{-6}$  M) for 24 h, and then the cellular and medium GH levels were determined. As shown in Fig. 6, corticosterone significantly increased the cellular GH level but not as much the medium GH level.

We also examined the morphological changes of MtT/S cells on glucocorticoid stimulation. Confocal laser microscopic observation showed that immunoreactive GH in the control MtT/S cells was mainly restricted to the



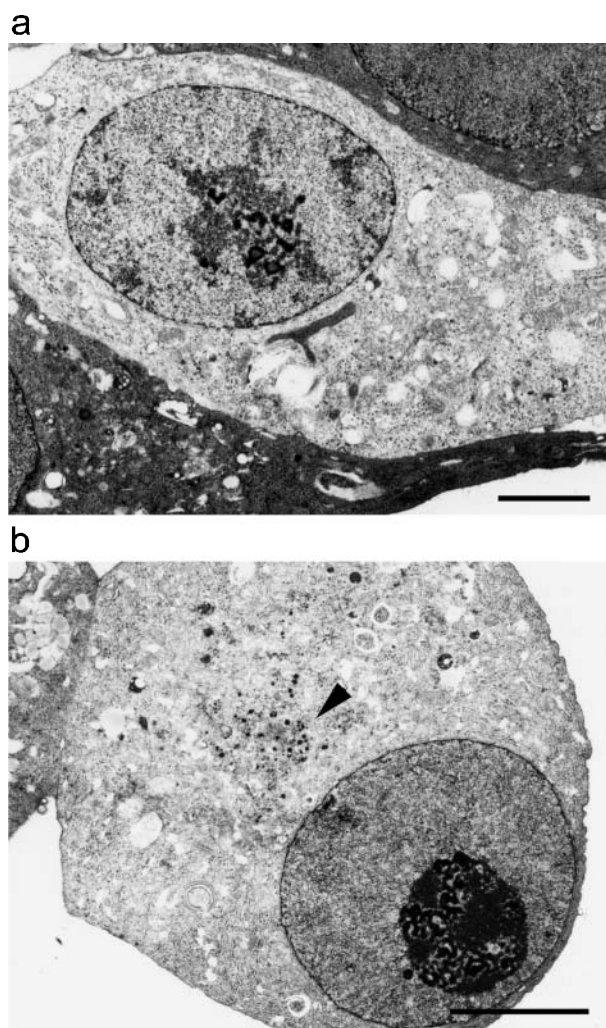
**Figure 4** Effects of RA and corticosterone (cor.) on GH secretion from MtT/E cells. MtT/E cells were incubated with RA ( $1 \times 10^{-6}$  M) for 5 days and then treated with corticosterone ( $1 \times 10^{-8}$  M) for 24 h in the presence of RA, and thereafter stimulated with GHRH for just 30 min. The medium GH levels were determined by RIA. The GH secretion from MtT/E cells was stimulated by RA. The results suggested that MtT/E cells responded to GHRH when treated with RA and corticosterone. Values are means  $\pm$  s.e. ( $n=3$ ). \* $P<0.05$  vs control.

cytoplasm adjacent to the nucleus, probably corresponding to the Golgi apparatus (Fig. 7A(a)). In contrast, glucocorticoid induced many dot-like GH immunopositive granules in the cytoplasm of MtT/S cells, as shown in Fig. 7A(b). The dramatic accumulation of secretory granules in the corticosterone-treated MtT/S cells was confirmed by electron microscopy, i.e. the secretory granules of MtT/S cells in the normal cultivation were not many (Fig. 7B(a)); however, many secretory granules were observed in the corticosterone-treated MtT/S cells (Fig. 7B(b)). These results suggested that corticosterone stimulated the accumulation of GH in the cytoplasm of MtT/S cells.

In order to examine the changes in the GHRH responsiveness of glucocorticoid-treated MtT/S cells, we incubated MtT/S cells in medium with or without corticosterone ( $1 \times 10^{-8}$  M) for 24 h. They were then changed to fresh medium and stimulated with GHRH ( $1 \times 10^{-9}$  M,  $1 \times 10^{-8}$  M and  $1 \times 10^{-7}$  M) or the vehicle for 30 min. As shown in Fig. 8, no response to GHRH was observed in the control group. In contrast, GHRH dose-dependently increased the medium GH level for MtT/S cells pretreated with corticosterone. Cell growth was inhibited dose-dependently by corticosterone treatment (Fig. 9).

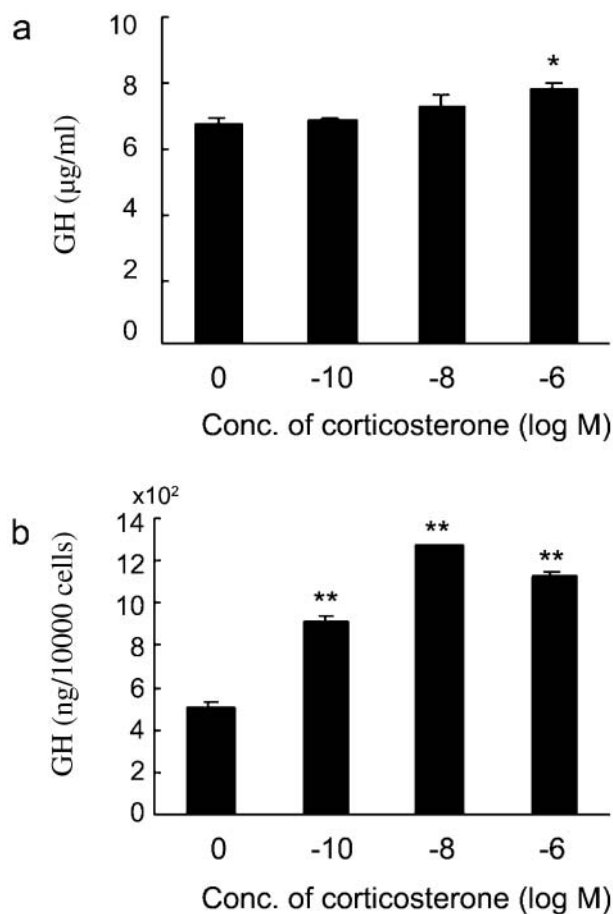
## Discussion

In order to study the mode of endocrine cell differentiation from progenitor cells, we were especially interested in



**Figure 5** Electron microscopical appearance of MtT/E cells. MtT/E cells (a) treated with RA had no secretory granules in their cytoplasm, but (b) glucocorticoid stimulation after RA treatment induced a small number of secretory granules near the Golgi area (arrowhead). Scale bars=2 $\mu$ m (a) and 3  $\mu$ m (b).

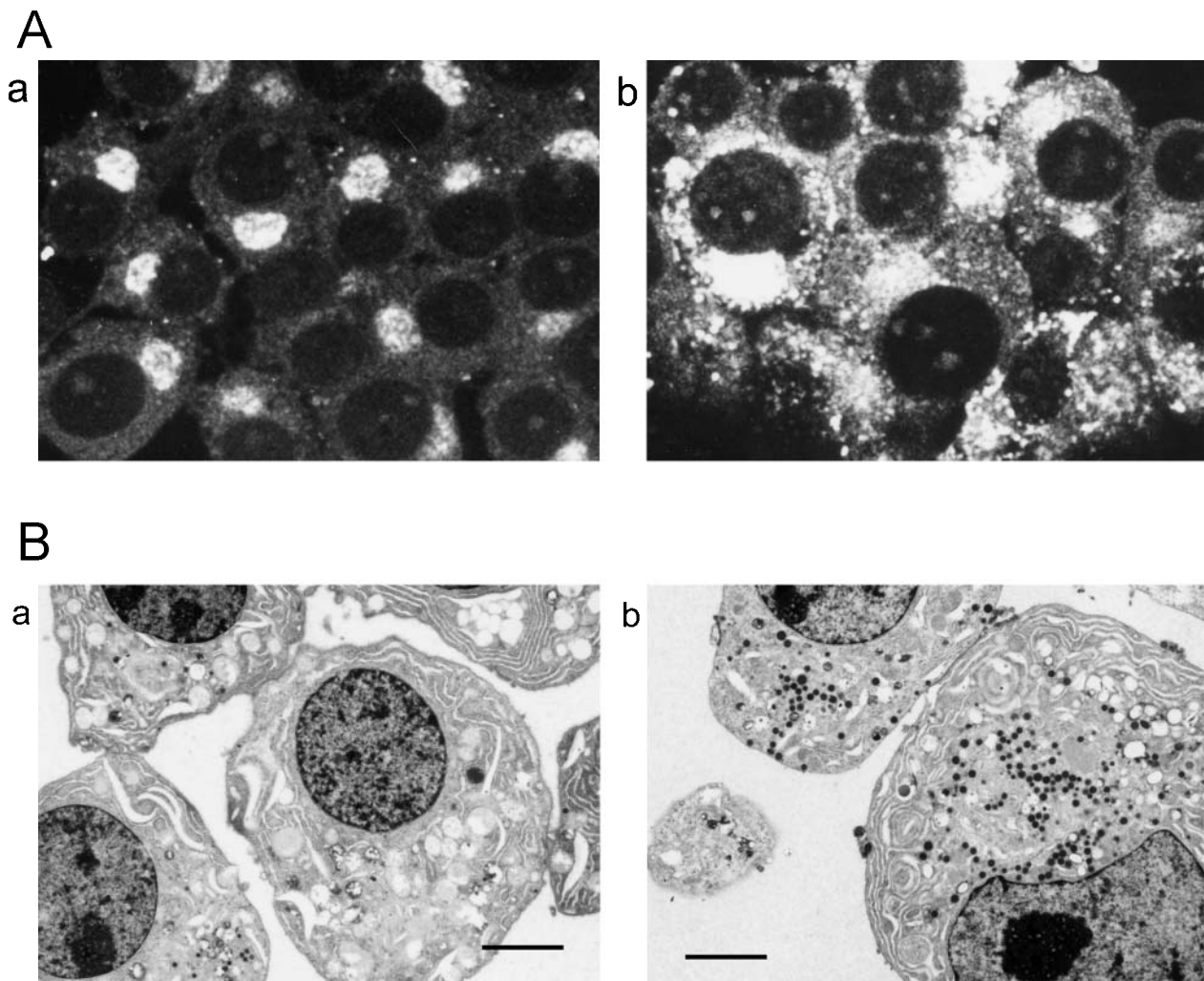
MtT/E cells. MtT/E cells were derived as a series of pituitary endocrine cell lines (Inoue *et al.* 1990). Most of these cell lines, such as MtT/S, MtT/Se and MtT/SM cells, are known to produce GH only or both GH and PRL; however, MtT/E cells do not produce any pituitary hormones. Our present data showed that MtT/E cells produced IL-6 and responded to PACAP. The morphological appearance of MtT/E cells was flat and spindle-shaped, and their proliferation was stimulated by bFGF. Interestingly, these characteristics of MtT/E cells resemble those of pituitary non-hormone-producing cells, such as folliculo-stellate cells. On the other hand, immunocytochemistry demonstrated that MtT/E cells were positive for Pit-1 protein in their nuclei, which suggests that MtT/E cells are committed to the Pit-1 cell lineage and have the



**Figure 6** Effect of concentration (Conc.) of corticosterone on GH cells. MtT/S cells were incubated with or without corticosterone ( $1 \times 10^{-10}$  M,  $1 \times 10^{-8}$  M and  $1 \times 10^{-6}$  M) for 24 h, and then the (a) medium and (b) cellular GH levels were determined. Corticosterone slightly increased the medium GH level, while the cellular GH level was significantly increased by corticosterone treatment compared with the control. Values are means  $\pm$  s.e. ( $n=4$ ). \* $P<0.05$ , \*\* $P<0.01$  vs control.

potential to differentiate into endocrine cells. Therefore, MtT/E cells were used in this study on early endocrine cell differentiation, and we clearly showed that RA induces the differentiation of MtT/E cells into GH-producing ones.

RA is known to induce the differentiation of many kinds of cells; however, our present study is the first showing that RA acts on non-hormone-producing cells among pituitary cells and induces GH cell differentiation *in vitro*. The tissue concentration of active RA is regulated by two enzymes, i.e. synthesizing (retinaldehyde dehydrogenase) and metabolizing RA (cytochrome P450) (Sakai *et al.* 2001). Retinaldehyde dehydrogenase type 2 is known to be expressed in the developing mouse pituitary gland as early as embryonic day 12.5 (Niederreither *et al.* 1997), i.e. a critical time of pituitary development. In

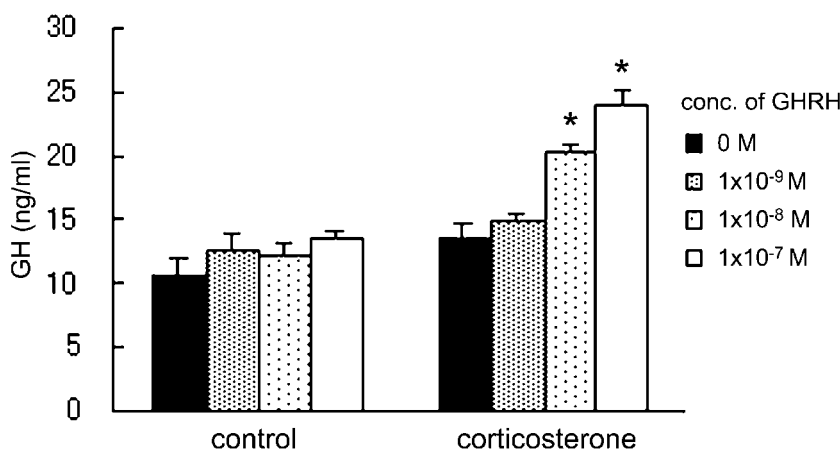


**Figure 7** Morphological effect of glucocorticoid on MtT/S cells. (A) Confocal laser microscopical appearance of immunoreactive GH localization of MtT/S cells. (a) Immunoreactive GH was localized in the Golgi apparatus. (b) In contrast, many dot-like immunopositive signals appeared in the cytoplasm, in addition to the Golgi apparatus, of corticosterone-treated MtT/S cells. (B) Electron microscopical appearance of MtT/S cells. Although MtT/S cells in the control medium had a small number of secretory granules in the (a) cytoplasm, (b) many appeared in the corticosterone-treated MtT/S cells. Scale bars=1μm.

In addition, it was previously reported that the RA-responsive element exists upstream of the Pit-1 (Cohen *et al.* 1999) and GH (Palomino *et al.* 1998) genes. Indeed, RA is known to induce Pit-1 mRNA transcription and act synergistically with dexamethasone to increase GHRH receptor expression (Cohen *et al.* 1999, Nogami *et al.* 2000). Therefore, it is suggested that RA may be involved in an early stage of GH cell differentiation through enhancement of GH and Pit-1 gene expression.

The inhibitory effect of RA on the proliferation of MtT/E cells also suggested that RA induces the differentiation of MtT/E cells into endocrine cells, which may show lower mitotic activity compared with progenitor cells. RA-stimulated MtT/E cells, however, did not respond to GHRH and no secretory granules were ob-

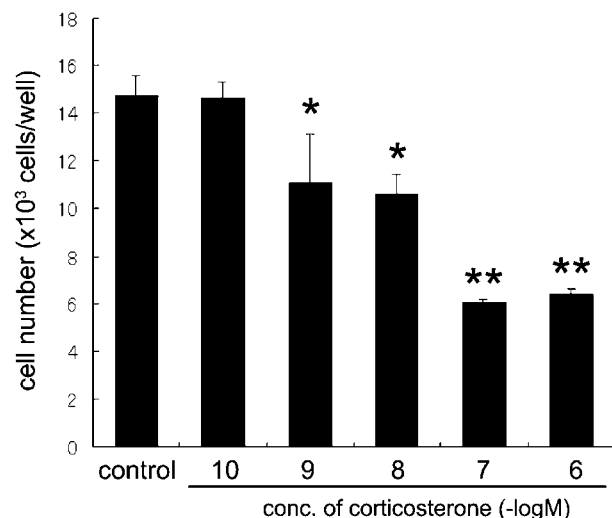
served in their cytoplasm. In order to obtain fully differentiated GH cells, we used glucocorticoid combined with RA, and found that MtT/E cells partially differentiated with this treatment, i.e. the cells responded to GHRH and a small number of secretory granules appeared. However, the response to GHRH and the number of secretory granules were low and different from those of mature GH cells in the pituitary gland. Although we cannot explain the reason, the GH cells derived from MtT/E cells with RA and glucocorticoid were not fully differentiated. For the full differentiation of GH cells from MtT/E cells, an unknown mechanism may be needed. If MtT/E cells are the progenitor cells of the Pit-1 lineage, there may be the potential to differentiate into PRL- or thyrotropin (TSH)-producing cells; however, we failed to differentiate



**Figure 8** Effect of corticosterone on the GHRH responsiveness of MtT/S cells. In the control group, MtT/S cells released GH into the culture medium with no response to GHRH, whereas MtT/S cells pretreated with corticosterone ( $1 \times 10^{-8}$  M) for 24 h showed a significant response to GHRH. Values are means  $\pm$  S.E. ( $n=4$ ). \* $P < 0.01$  vs control.

MtT/E cells into either PRL cells or TSH cells (data not shown). An unknown mechanism may be needed.

To induce fully differentiated GH cells *in vitro*, we used MtT/S cells. MtT/S cells are committed to GH cells; however, GH immunoreactivity is restricted to the Golgi apparatus and MtT/S cells respond incompletely to GHRH (Inoue *et al.* 1990). This suggests that MtT/S cells are immature GH cells. When we stimulated MtT/S cells with glucocorticoid, we found that the medium GH



**Figure 9** Effect of concentration (conc.) of corticosterone on MtT/S cell proliferation. MtT/S cells were incubated in a 24-well culture plate with or without corticosterone ( $1 \times 10^{-10}$  to  $10^{-6}$  M) for 4 days. After incubation, the cell number was determined with a hemocytometer. Corticosterone significantly suppressed cell proliferation in a dose-dependent manner from  $1 \times 10^{-9}$  M. Values are means  $\pm$  S.E. ( $n=4$ ). \* $P < 0.05$ , \*\* $P < 0.001$  vs control.

and the cellular GH levels were significantly increased. Secretory granules in the cytoplasm of MtT/S cells also dramatically increased with the corticosterone treatment. Immunocytochemistry clearly showed that the glucocorticoid-induced secretory granules are immunopositive for GH. These morphological characteristics are exactly the same as those of the somatotroph in the pituitary gland. Additionally, GHRH responsiveness is induced by glucocorticoid. These results may indicate that glucocorticoid induces full differentiation of GH cells. In support of our finding, it has been reported that glucocorticoid increases the GHRH receptor level in the pituitary (Seifert *et al.* 1985) and MtT/S cell line (Nogami *et al.* 1999). Interestingly, it has been reported that MtT/S cells have two isoforms of GHRH receptors, but that only the short isoform is active (Miller *et al.* 1999). Therefore, we cannot neglect the possibility that glucocorticoid stimulates the expression of the active GHRH receptor form.

The population of GH cells in the anterior pituitary gland, as revealed by immunocytochemistry, increases mainly between embryonic days 18 and 19, when the serum corticosterone level is elevated. However, with dexamethasone stimulation, GH cell appearance was earlier than in control fetuses (Nogami & Tachibana 1993, Nogami *et al.* 1995). This report indicates the existence of premature GH cells in the process of pituitary development, and glucocorticoid may be concerned in GH cell maturation *in vivo*. The present study showed that corticosterone did not stimulate GH cell proliferation but slightly suppressed MtT/S cell proliferation. Therefore, glucocorticoid may stimulate GH production in GH cells or its progenitor cells, not through proliferation of GH cells, but through maintenance of GH cell function in co-operation with other factors such as thyroid hormone and GHRH.



In this connection, thyroid hormone is known to enhance the GH transcription rate by itself or synergistically with glucocorticoid (Yaffe & Samuels 1984, Nyborg *et al.* 1984). It has also been noted that a point mutation of the GHRH receptor gene leads to a decrease in GH cells (Godfrey *et al.* 1993, Lin *et al.* 1993). In addition, Dean & Porter (1999) recently demonstrated that corticosterone and GHRH co-operatively regulate GH cell differentiation and GH secretion at specific stages of embryonic development. In support of their findings, immunoreactive GHRH fibers have been demonstrated in the median eminence at embryonic day 18 (Ishikawa *et al.* 1986, Jansson *et al.* 1987). These reports strongly suggest that corticosterone regulates GH cell development in cooperation with other factors.

In conclusion, we consider that MtT/E cells and MtT/S cells correspond to progenitor cells and premature GH cells respectively. The present study has shown that the progenitor cells need RA priming to respond to glucocorticoid. RA induces the differentiation into immature GH cells from non-hormone-producing progenitor cells, and then glucocorticoid induces mature GH cells from premature GH cells. We therefore propose that GH differentiation is caused by multistep processes.

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