Immunoreactive Pit-1 Protein in Hyperplastic Pars Intermedia Induced by Calcitonin of the Rat Pituitary Gland

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Abstract. To elucidate the effects of synthetic salmon calcitonin (sCT) on the cells in the rat pituitary gland, we histopathologically and immunohistochemically examined the early changes after 4 or 13 weeks treatment with sCT 120 IU/kg. Focal proliferative lesions of the anterior pituitary glands were consistently found after treatment with sCT for 13 weeks. Histologically, the cells with the focal proliferative lesions were classified into the following three groups: 1) enlarged basophilic cell focus, 2) vacuolated cell focus and 3) chromophobe cell focus. These focal proliferative lesions had positive staining only for the alpha-subunit and failed to show Pit-1 protein immunoreactivity. The sCT treatment also increased the thickness of the pars intermedia. Hypertrophy of the pars intermediate cells was characteristically seen. Furthermore, Pit-1 protein immunoreactivity was clearly detected in the nuclei of the hyperplastic pars intermediate cells. All pars intermediate cells were equally stained by alpha- or beta-MSH and beta-endorphin in both vehicle- and sCT-treatment. No difference was seen. These findings strongly suggest a very close relationship between Pit-1 protein immunoreactivity and cellular proliferation induced by sCT.

Key words: Calcitonin, Pituitary gland, Alpha-subunit, Pit-1, Rat

(RECENTLY, we reported that administration of synthetic salmon calcitonin (sCT) for 1 year resulted in an increase in pituitary gland hyperplasia and adenomas in Sprague-Dawley rats [1]. Furthermore, we demonstrated that only the alpha-subunit was stained by the immunoperoxidase method in the pituitary tumors induced by sCT [1]. Therefore, we postulated that sCT-induced pituitary tumors were endocrinologically non-functioning and alpha-subunit-producing tumors [1].

Some pituitary tumors are not associated with the hypersecretion of hormones. Because a specific tumor marker has yet to be found, the diagnosis of non-functioning chromophobe adenoma is not made in these patients until the tumors are so large that they produce visual loss and other compressive symptoms. Similarly, it is difficult to assess the completeness of pituitary surgery and to monitor these patients after radiotherapy [2-6]. Their follow-up examination to evaluate tumor recurrence is limited to visual field changes or radiographic abnormalities [2-6]. In the present study, in order to confirm the biological significance of sCT-induced rat pituitary tumor, we histopathologically and immunohistochemically examined the early changes after 4 or 13 weeks treatment with sCT.

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Materials and Methods

Animals and tissue preparation

Male Sprague-Dawley rats were purchased from Charles River Japan Inc. (Atsugi, Japan) at the age of five weeks. The animals were kept in a barrier-divided room, which was maintained at a temperature of 22±2°C with a humidity of 60±10%. The room was ventilated 23 times per h and provided with 12 h of light (from 8:00 to 20:00). The animals were housed in individual plastic cages (CLEA Japan Inc.). Solid food (CE-2, CLEA Japan Inc.) and tap water were available to all animals ad libitum. One week was allowed for the animals to adjust to laboratory conditions.

The sCT (Teikoku Hormone Mfg. Co., Ltd., Kawasaki) is a type I form of naturally produced salmon calcitonins [7], and has a potency of about 5,000IU/mg. The sCT was dissolved in an acetic acid buffer (pH 4.0).

Twenty animals were used as untreated controls. Twenty rats each received daily administration of vehicle or sCT (120IU/kg/day) by subcutaneous injection for 4 or 13 weeks. Each rat was sacrificed on the day after the last injection under ether anesthesia. The pituitary glands were removed immediately.

Histopathological examination

The pituitary glands were fixed in 0.1 M phosphate-buffered 10% formalin, embedded in paraffin, mounted and stained with hematoxylin and eosin (HE).

Immunohistochemical staining

Indirect immunoperoxidase method was used to stain the 4 μm paraffin sections. Primary antibodies human (h) ACTH (1:500), hGH (1:100) and hPRL (1:500) were obtained from Dako (Carpinteria, CA). Rat (r) LHbeta (1:100), rTSHbeta (1:100), rFSHbeta (1:100) and alpha-subunit of glycoprotein hormone (1:100) were gifts from the National Institute of Diabetes, Digestive and Kidney Diseases (NIDDK, MD) and r alpha-MSH (1:2000), r beta-MSH (1:2000) and r beta-endorphin (1:4000) were obtained from UCB-Bioproduct (Belgium). These antisera were incubated with the paraffin sections at room temperature for 30 min. The sections were then incubated with horseradish peroxidase (Sigma Chemical Co., St. Louis, MO)-labeled Fab fragment of goat IgG against rabbit IgG for 30 min at room temperature. After the completion, the sections were treated with Graham-Karnovsky's reaction medium [8], which contained 20 mg% 3,3'-diaminobenzidine (DAB, Wako Pure Chemical Industries, Osaka) and 0.005% hydrogen peroxide in 0.05 M Tris-HCl buffer, pH 7.6, for 5 to 10 min at room temperature. The sections were then counterstained for nuclei with 1% methyl green dissolved in veronal acetate buffer, pH 4.2.

Anti-rPit-1 antibody was obtained from Santa Cruz Biotechnology Inc. After deparaffinization, the endogenous peroxidase was blocked with 0.1% hydrogen peroxide-methanol for 30 min at room temperature and the sections were then pretreated by microwaving for 10 min in 0.01 M citrate buffer, pH 6.0, for antigen retrieval. After washing with 0.01 M phosphate-buffered saline (PBS), the sections were incubated for 1 h at room temperature with rabbit polyclonal antibody against rat Pit-1 protein diluted at 1:200. After washing with 0.01 M PBS, the sections were covered with biotin-conjugated goat anti-rabbit IgG for 1 h, washed again and then treated with streptavidin-biotin-peroxidase complex (Histofine SAB-PO(R)Kit, Nichirei, Tokyo) for 1 h. After washing with 0.01 M PBS, the immunoperoxidase staining was performed as described above.

Statistical analysis

Data was expressed as mean±S.D. Statistical analysis was conducted with multiple comparison test after one-way analysis of variance (ANOVA) of the data for the pars intermediate cell area.

Results

1) Histopathological findings

Focal proliferative lesions of the pars distalis in the pituitary glands were hardly seen after treatment with sCT for 4 weeks. In contrast, focal proliferative lesions were consistently found after treatment with sCT for 13 weeks. Focal proliferative lesions were
characterized by an increase in the number of a single cell type within a defined area that blends imperceptibly with the surrounding parenchyma. Compression was not present. Histologically, the cells which make up the focal proliferative lesions were classified into the following groups: 1) enlarged basophilic cell focus (Fig. 1A), 2) vacuolated cell focus (Fig. 1B) and 3) chromophobe cell focus (Fig. 1C). The sCT treatment also increased the thickness of the pars intermedia without inducing any disorder of the lobular structure or degeneration of the pars intermediate cells. Hypertrophy of the pars intermediate cells were clearly detected (Figs. 2A, B). Compared with that in the vehicle-treated rats, the mean cell size was significantly increased in the rats treated with sCT for 4 weeks (Table 1).

2) Immunohistochemical findings

Three types of focal proliferative lesions induced by sCT in the pars distalis failed to show immunoreactivity for any of the peptide hormones in-

Fig. 1. Light microscopic view of focal proliferative lesions induced by sCT in the rat anterior pituitary gland. A: Enlarged basophilic cell focus (arrows). B: Vacuolated cell focus (arrows). C: Chromophobe cell focus (arrows). HE ×200 (A–C).

Fig. 2. Light microscopic view of pars intermedia. A: Vehicle control. B: The pars intermediate cells in the rat given sCT demonstrated diffuse hypertrophy. HE ×200.
including ACTH, PRL and GH. Furthermore, no immunoreactivity was noted for the beta-subunits of the glycoprotein hormones, FSH, LH and TSH. In contrast, all three types of focal proliferative lesions induced by sCT had positive staining for the alpha-subunit (Fig. 3A) and negative for Pit-1 protein (Fig. 3B). All pars intermediate cells were equally stained by antisera against alpha- (Figs. 4A–D) or beta-MSH (Figs. 5A–D) and beta-endorphin (Figs. 6A–D) in both vehicle- and sCT-treated rats. No difference was seen between the vehicle and sCT-treated rats in the immunostaining for alpha- or beta-MSH and beta-endorphin. The majority of the nuclei in the hypertrophic pars intermedia induced by sCT had positive staining for Pit-1 protein (Fig. 7).

**Discussion**

Focal proliferative lesions induced by sCT were

**Table 1.** The mean pars intermediate (PI) cell area in rats treated subcutaneously with sCT for 4 or 13 weeks.

<table>
<thead>
<tr>
<th>sCT</th>
<th>Mean PI cell area ± SD (μm²)</th>
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<tr>
<td>(IU/kg/day)</td>
<td>n</td>
</tr>
<tr>
<td>0</td>
<td>10</td>
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<tr>
<td>120</td>
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**P < 0.01 vs vehicle control

![Fig. 3. Immunostaining for alpha-subunit (A) and Pit-1 protein (B) in focal proliferative lesions induced by sCT in the rat anterior pituitary gland. A: Focal proliferative lesions induced by sCT are positively stained for only alpha-subunit. B: Focal proliferative lesions induced by sCT failed to show immunoreactivity for Pit-1 protein. Peroxidase-labeled antibody method ×200 (A, B).](image1)

![Fig. 4. Immunostaining for alpha-MSH in pars intermedia. A: Vehicle control (13 weeks). B: sCT-treatment for 13 weeks. Peroxidase-labeled antibody method ×150 (A, B).](image2)
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Fig. 5. Immunostaining for beta-MSH in pars intermedia. A: Vehicle control (13 weeks). B: sCT-treatment for 13 weeks. Peroxidase-labeled antibody method ×150 (A, B).

Fig. 6. Immunostaining for beta-endorphin in pars intermedia. A: Vehicle control (13 weeks). B: sCT-treatment for 13 weeks. Peroxidase-labeled antibody method ×150 (A, B).

Fig. 7. The majority of the nuclei in the hypertrophic pars intermedia induced by sCT are positivity stained for Pit-1 protein. Peroxidase-labeled antibody method ×100.

stained only for alpha-subunit by the immunoperoxidase method. The mature pituitary is composed of six different types of cells, five in the anterior lobe and one in the intermediate lobe, that appear sequentially during development and are easily distinguishable by the hormone secreted [9]. The glycoprotein hormone alpha-subunit is the first hormone subunit to be expressed in the developing mouse pituitary on embryonic day 11.5 (e11.5), followed by anterior pituitary POMC on e12, TSH on e14, intermediate lobe POMC for MSH synthesis on e14.5, GH and PRL on e15.5, LH on e16.5, and FSH on e17.5 [10-12]. In the rat pituitary, alpha-subunit appears on embryonic day 11 (e11) as the first anterior pituitary marker [9], and alpha-subunit expression is restricted to the anterior most pituitary
cells at this stage [9]. Recent studies on the ontogeny of pituitary cell development in the rat indicate that expression of the alpha-subunit gene precedes that of the other hormone genes by several days and in fact occurs early in the formation of Rathke's pouch [9]. Thus, the alpha-subunit producing cells may be relatively primitive pituitary cells with some characteristics of stem cells.

In the present study, focal proliferative lesions induced by sCT failed to show Pit-1 protein. In rodents and humans, differentiation and/or maintenance of somatotroph, lactotroph and thyrotroph phenotypes are dependent on expression of functional Pit-1 gene mutation that results in hypopituitarism [13-15] and hypoplasia of somatotrophs, lactotrophs, and thyrotrophs [13]. Moreover, it was shown that Pit-1 antisense oligonucleotides not only block GH and PRL transcription, but also inhibit [3H]thymidine incorporation by somatotroph and lactotroph cell lines, suggesting that Pit-1 may regulate DNA replication and cell proliferation [16]. We found that the sCT treatment produced an increase in the thickness of the pars intermedia, and that the mean cell size was significantly increased. Horiuchi et al. [17] demonstrated that the daily administration of sCT (120 IU/kg) for 3, 7 and 14 days produced 2.8-, 2.6-, and 2.3-fold increase, respectively, in the rate of cell proliferation as estimated by BrdU labeling. This increase after sCT treatment is thought to be indicative of hyperfunction of the pars intermedia, since haloperidol, a D-2 dopamine receptor antagonist which enhances pars intermedia function, increases and bromocriptin, a D-2 agonist which inhibits the pars intermedia, decreases the rates of cell proliferation as determined by the uptake of [3H] thymidine or by the mitotic index [18]. In fact, Pit-1 protein immunoreactivity was detected in the nuclei of the hyperplastic pars intermediate cells. The reason for the markedly increased Pit-1 immunoreactivity in the pars intermedia is unclear. A recent study using antisense oligonucleotide analysis indicated that Pit-1 was involved in proliferation in a rat pituitary cell line [16], suggesting that Pit-1 may have a role in cell proliferation and tumor development. In contrast, all pars intermediate cells were equally stained by alpha- or beta-MSH and beta-endorphin in both vehicle and sCT-treatment. No difference was seen. Based on our findings and these facts, the morphological changes after sCT treatment strongly suggest that sCT enhances the activity of pars intermediate cells of the rat pituitary gland.

Recent studies with transgenic mice deficient in P27 has shown that this protein inhibits proliferation in some tissues such as the thymus, pituitary, and spleen leading to hyperplasia of these tissues and an increase in general body weight, despite normal levels of serum GH and insulin-like growth factor-1 when P27 was absent [19]. Interestingly, the pituitary of transgenic mice with P27 gene deletion showed ACTH cell hyperplasia from the intermediate lobe. Future studies must determine the mechanistic relationships between P27 expression and hyperplastic pars intermediate cells induced by sCT.

It is well documented that calcitonin is involved in calcium regulation in mammals, mainly by inhibiting osteolysis. The sCT is a peptide hormone composed of 32 amino acid residues. In mammals, maintenance of the extracellular calcium ion concentration within narrow limits is essential for the function of many tissues. In addition, calcium ions are also essential for the secretory activity of endocrine and exocrine cells. Furthermore, calcium is an important and ubiquitous regulator of various intracellular enzyme activities. As focal proliferative lesions of the anterior pituitary glands are induced by sCT, we cannot determine from the present study whether the effects of sCT are direct or indirect. It is possible that one of the many systemic physiological effects of sCT leads indirectly to the proliferation of a subset of a pituitary cell-type. It is notable that calcitonin is produced in large amounts in the posterior hypothalamus and median eminence, raising the possibility that calcitonin may normally exert effects on the hypothalamus-pituitary axis [20, 21]. Calcitonin receptors have been identified in the hypothalamus, and lower numbers of receptors are found in the pituitary gland [21]. Infusion of calcitonin in rats has been reported to cause a reduction in several pituitary hormones including GH, PRL, LH, FSH, and TSH [22]. The mechanism for these effects has not been elucidated and it remains to be shown whether sCT is acting at the hypothalamic or pituitary level. In dispersed pituitary cells, calcitonin has been shown to inhibit TRH stimulation of PRL secretion [23], suggesting that some of the effects of sCT may occur directly at the level of the pituitary.
The pars intermedia is known to be regulated primarily by dopaminergic neurons emanating from the arcuate nucleus of the hypothalamus and terminating directly on pars intermediate cells [24]. Thus, pharmacological manipulation with dopaminergic drug has been shown to elicit changes in the rat pars intermediate cells morphology, secretion of POMC-derived peptide, POMC mRNA content, and rate of cell proliferation [25-27]. Future studies must determine the mechanistic relationships between POMC or Pit-1 gene expression and cellular proliferation induced by sCT.

References


