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Exogenous Calcitonin Suppresses Growth Fraction of Thyroid C Cells

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Calcitonin (CT) is a peptide hormone synthesized and secreted by the parafollicular (C) cells of the thyroid. Little is known about the mechanisms controlling proliferation of C cells by other humoral factors including CT, and there is no report that CT suppresses C cell proliferation. The effects of short-term administration of CT on C cell growth fraction were analyzed using BrdU and CT double immunohistochemical method in the rat thyroid. Continuous administration of 0.4 IU/kg and 40 IU/kg synthetic salmon CT for 14 days induced dose-dependent suppression of BrdU labeling number of C cells without decrease of C cell to the total thyroid follicular cell ratio. These results suggest that exogenous CT may induce negative feedback to C cell proliferation in the thyroid.

Key words: calcitonin, C cell, growth fraction, rat, thyroid

I. Introduction

Calcitonin (CT) is a peptide hormone synthesized and secreted by the parafollicular (C) cells of the thyroid [2, 18]. Besides the hypocalcemic effect of CT, it is well established that CT administration shows regulatory effects on a variety of hormone secretion. Secretion of gastrin [6], insulin [3], GH [16], TSH [15], prolactin [20], and prostaglandin [4] were suppressed by the preinfusion of CT. On the other hand, hormone secretions were stimulated by CT infusion in the following hormones, such as ACTH [14], somatostatin [5, 10, 17], and beta-endorphin [14] secretions. Auto-regulation of CT secretion has been suggested as a feedback mechanism by some researchers using porcine thyroid slices [1, 21]. Morimoto *et al.* have supported the presence of a negative feedback mechanism of CT secretion, when salmon CT was administered to rat [19]. However, little is known about the mechanisms controlling proliferation of C cell by CT. Suppression of the C cell population by long-term administration of CT was earlier reported from our laboratory in the rat. In that 53 week CT administration study on rats and C cell tumors and its precursor lesions were significantly decreased [13]. In this study, the short-term effects of CT administration on CT synthesis and cell cycle of C cells of rat thyroid were analyzed.

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II. Materials and Methods

Materials

Synthetic salmon calcitonin (TZ-CT) was purchased from Teikoku Hormone Mfg. Co. Ltd. (Tokyo, Japan).

Sixty of nine-week old male Wistar-Imamichi rats (Institute for Animal Reproduction, Saitama, Japan) were used. The animals were kept in wire cages on a standard laboratory diet (CE-2, CLEA, Co., Tokyo, Japan) and tap water *ad libitum*. The rats were randomly divided into three groups (n=20/group): B group were injected subcutaneous treated with daily dose of 0.4 IU/kg CT, C group were with 40 IU/kg daily for 14 days, and A group were given only vehicle. To monitor cellular DNA synthesis, BrdU (5-bromo-2'-deoxyuridine; Sigma, Inc., St. Louis., MO) was administered continuously by using osmotic miniature pump (Model 2001, ALA Corp., Palo Alto, CA) from the first experimental day to the last sacrificed day continuously to all three groups. Osmotic miniature pump was filled with BrdU (120 mg/ml; Sigma, Inc.). An osmotic miniature pump was inserted on the first day of experiment in the abdominal cavity under anesthesia. The BrdU solution was released at a rate of 1 μl/hr (120 μg BrdU/hr) for maximum of 14 days. After one hour of CT administration, each five animals were sacrificed at day 1, 3, 7, and 14 under ether anesthesia. After perfusion-fixation by 70% ethanol through left ventricle of the heart, the thyroids and kidneys were excised following immersion fixation in 70% ethanol for 6 hr at 4°C. For thyroid, double immunostaining of BrdU and CT was performed, and for

kidney, BrdU alone.

BrdU/CT double immunostaining

BrdU incorporation into proliferating cells was determined immunohistochemically on 5 μm paraffin sections. Endogenous peroxidases were neutralized by incubating sections in 0.3% H_2O_2/methanol for 30 min. DNA was denatured in tissue sections by incubation in aqueous 2 N HCl for 60 min at room temperature (RT). Anti-BrdU antibody (Becton Dickinson, CA) (1:30 dilution) was applied for 45 min at RT. Biotinylated horse antimouse IgG was applied and then avidin-biotin-peroxidase complex was applied for 30 min. Antibody binding sites were visualized using a 0.02% 3,3-diaminobenzidine peroxidase, 0.5% cobalt-chloride and 0.005% H_2O_2/0.05 M Tris-HCl buffer (pH 7.6). After that, the slides were washed with 0.1 M glycine-HCl buffer (pH 2.2) for 60 min at RT followed by CT immunohistochemistry. For CT immunohistochemistry anti-synthetic human CT rabbit serum (Dako Japan, Co., Kyoto, Japan) was used followed by the second antibody (HRP labeled anti-rabbit antibody, Dako Japan). After washing, DAB in PBS solution was used for visualization of the immunoreactions for CT.

Evaluation of BrdU cumulative labeling

C cell growth fraction was evaluated by counting BrdU-positive nuclei on immunostained sections under microscope. Optical photomicrographs from nine fields of thyroid (approximately 200 C cells were examined for BrdU labeling) and from three fields of renal outer cortex tubular epithelium taken at a magnification of ×100 were used for counting BrdU-positive cells. They were expressed in percentage.

Statistical analysis

Student's t test was used to compare data. A P value of <0.05 was considered statistically significant.

III. Results

Figure 1 shows double immunostaining for CT and BrdU on thyroid from 40 IU and 0 IU/day for 14 days rat thyroid. Those cells which have cytoplasm positively for DAB (brown) and nuclear positively for Cobalt-DAB (black) were regarded as C cells which entered in S phase fraction during experimental period, while only nuclear staining was regarded as follicular cell in S phase fraction. Administration of CT 0.4 IU/kg and 40 IU/kg for 14 days induced dose-dependent decrease of both BrdU labeled C cell ratio to total C cells (Fig. 2A). In order to remove the other unknown factors in this experiment such as accuracy of infusion by osmotic pump, the BrdU labeled C-cell to BrdU labeled renal epithelial cells ratio was calculated. The results also indicated CT suppressed the labeled C cell/renal cell percentage in dose dependent manner and a statistically significant difference between the CT treatment and control (Fig. 2B).

IV. Discussion

The hormone secretion of most endocrine organs is regulated by a negative feedback system by which synthe-

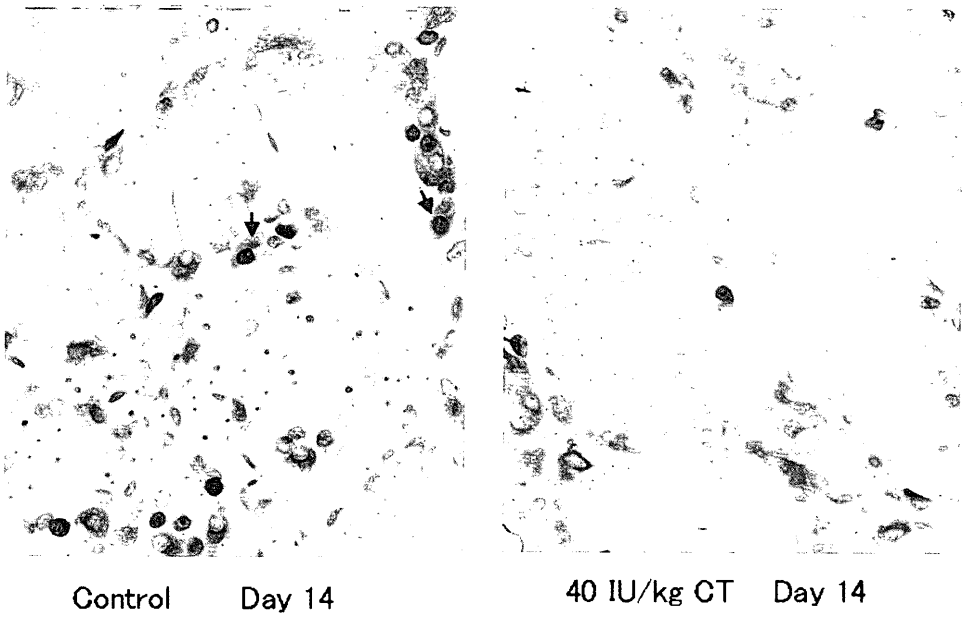

Fig. 1. BrdU/CT double staining in thyroids of rat treated with 0 and 40 IU/Kg CT daily for 14 days. Immunoreactivity for CT is visible in cytoplasm of C-cell, and BrdU positive cells, which entered S phase stage during the experiment, are stained in nuclei (arrows); magnification, ×370. **A:** Control rat thyroid at 14 day, **B:** 40 IU/kg of CT administration at 14 day.

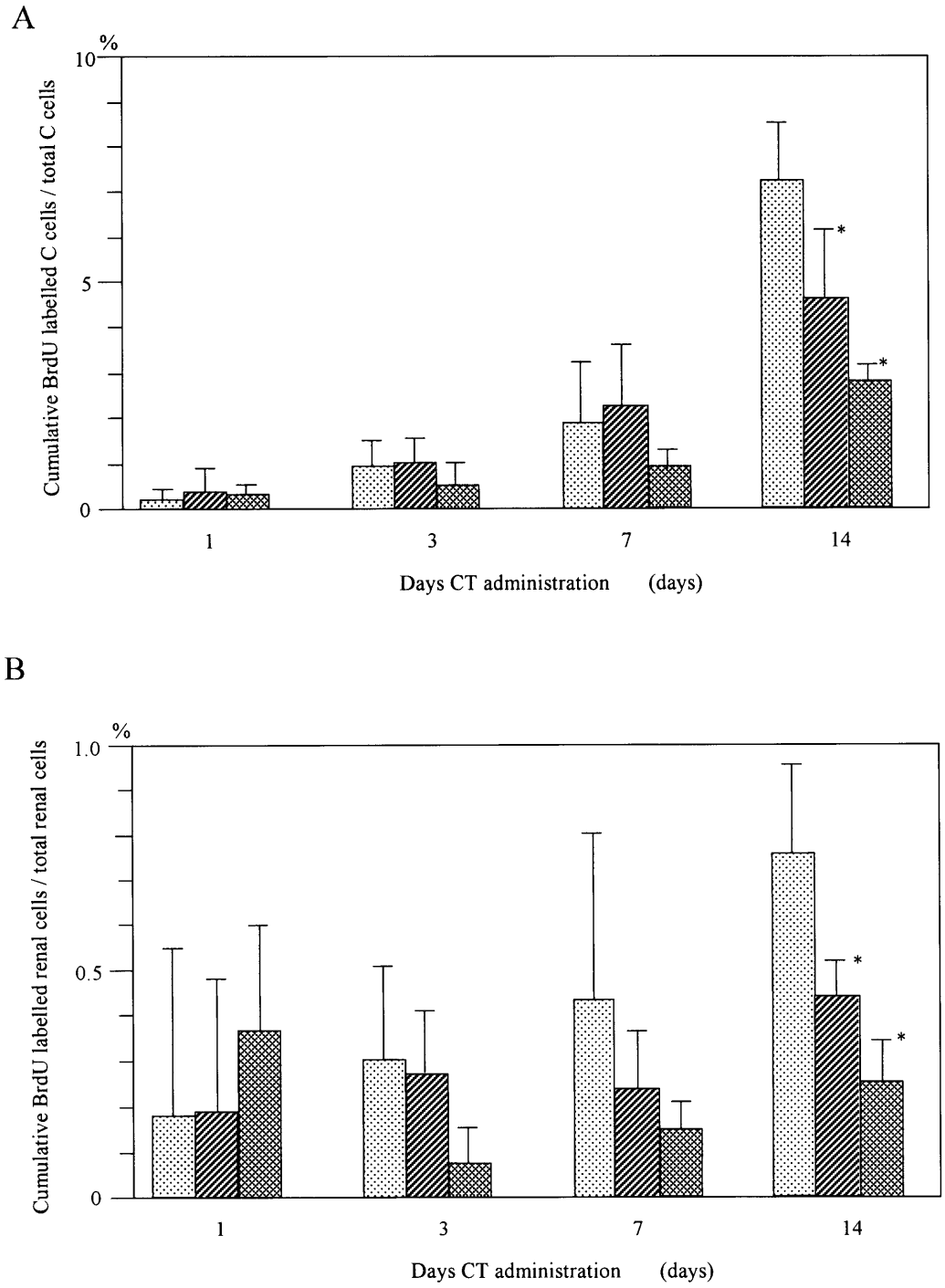

Fig. 2. Effects of various term administration of CT on proliferation of C cell. Five rats each were treated with 0 (▒), 0.4 (▨) and 40 (▩) IU/kg CT daily and simultaneously BrdU was administered continuously to all rats. After one hour of CT administration, each animal was sacrificed at day 1, 3, 7 and 14. Cumulative BrdU positive C cell to total C cells ratio (**A**) and BrdU positive C cells to total C cells ratio/BrdU positive renal cells to total renal cells (**B**) were measured. The values of the ratios shown are the average±SD of five samples.

sis, secretion of hormone and cell growth are controlled. Feedback regulation mechanisms of C cells and CT secretion have been assumed for CT in tissue culture studies. Ross *et al.* have reported that C cell function is regulated by serum Ca^{2+} level [22]. However, Morimoto *et al.* have reported that administration of CT in the rat rapidly inhibited CT secretion [19] without significantly changing plasma Ca^{2+} levels, and therefore CT treatment probably suppresses C cell function via negative feedback. We have reported that long-term CT administration suppressed the C

cell population without morphologic changes of C cells in rats [13], and that ipriflavone induced CT *de novo* syntheses and secretion in rat thyroid [23].

In the present study, short-term administration of CT was examined and showed decreased S phase fraction of C cell in two different doses using immunohistochemical method. We have also found that administration of CT for a short term caused dose-dependent reduction of CT and CT-mRNA in the rat thyroid under preparation (Takekoshi *et al.*). Whether exogenous CT induces negative feedback to the proliferation of C cells directly through calcitonin receptor (CTR) on C cells or indirectly via Ca^{2+} or the other factors was not answered by these results.

It is reasonably certain that CT acts directly on C cells through CTR and causes this suppression of C cell proliferation because CTR mRNA has been expressed in human medullary thyroid carcinoma tissues and the medullary thyroid carcinoma cell line, TT cell [7–9]. Izumi *et al.* have showed that osteoclasts on the bone surface express CTR by immunohistochemistry [12]. It is well known that down-regulation of CTR expression in the osteoclast is induced by high dose CT infusion in the osteoclast, which is known as the "escape phenomenon" of CTR [11]. CT and CTR on C cells of the thyroid may serve as a good experimental model to study this escape phenomenon of CTR. We hypothesized that this phenomenon may also occur in C cells. Further investigation will be needed to elucidate the detailed mechanism between CT and C cell proliferation.

In this study CT administration was carried out only for 14 days, this is because an osmotic miniature pump can only deliver for up to 14 days maximum. A subacute study, between 14 days to one year, may be necessary to explain C cell growth suppression in acute phase and suppression of C cell tumor in chronic phase and their respective mechanisms.

V. References

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