Immunohistochemical Alterations on Thyroid C Cells of Rabbits Treated with Vitamin D₃

Hiroyuki OKADA, Keigo YOROZU, Yutaka CHIHAYA, and Kiyoshi MATSUKAWA

Department of Veterinary Pathology, School of Veterinary Medicine, Rakuno Gakuen University, Ebetsu, Hokkaido 069, Japan
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Rabbits [4,5] and cattle [1] given excessive doses of vitamin D have developed systemic soft-tissue mineralization, particularly in aorta, heart, and lungs. It has been reported that the daily administration of vitamin D promotes hyperplasia and hypertrophy of C cells in cows [2], dogs [7] and rats [3, 15, 16]. The effect of hypercalcemia to C cells has been also reported immunohistochemically in various animals [9, 11, 12, 18], but not in rabbits. The purpose of the present study is to investigate the alteration of immunoreactivities for calcitonin, calcitonin gene-related peptide (CGRP), somatostatin, chromogranin A (CGA), and neuron-specific enolase (NSE) in rabbit C cells after treatment with excessive doses of vitamin D₃.

Fourteen male New Zealand white rabbits, 1 to 1 and half years of age and approximately 3 kg in weight, were used. Eleven of these rabbits were injected 2 times (Group A; 3 rabbits, Group B; 2 rabbits) or 4 times (Group C; 4 rabbits, Group D; 2 rabbits) intramuscularly with vitamin D₃ (diphyral D₃=1000, Duphar B. D., Amsterdam, Holland) of 40,000 IU/kg at 3 days intervals. They were sacrificed 4 days (Group A, C) or 18 days (Group B, D) after final vitamin D₃ injection (Fig. 1). Other three of these rabbits were used as control. The thyroid glands were fixed in Bouin’s or 10% formalin solution for at least 48 hr. They were embedded in paraffin. Sections were stained with hematoxylin and eosin (HE). Immunohistochemical staining was performed by the indirect method using specific rabbit antisera (Table 1). For comparison, sections of thyroid glands of rats were also stained for CGA and NSE. For control, sections were incubated with non-immune rabbit serum (Dakopatts, Denmark) and phosphate buffered saline (PBS) instead of the primary antisera.

In normal rabbits, it was difficult to distinguish C cells from follicular epithelial cells in HE sections (Fig. 2a). Calcitonin-immunoreactive cells regarded as C cells were located in the intrafollicular area as a single element or a small cluster (Fig. 2b). C cells were oval or elongated in shape and frequently having long cytoplasmic protrusions. They were concentrated in the central portion of both lateral lobes of the thyroid and mainly located in the intrafollicular position. Almost all calcitonin-positive cells stained intensely with both CGRP and somatostatin antisera (Figs. 2c, 2d). The immunoreactions for CGA and NSE were negative. On the other hand, almost all C cells of rats were intensely or moderately positive to CGA and weakly immunoreactive to NSE antisera [19]. All control sections and follicular epithelial cells revealed negative for non-immune rabbit serum and PBS.

In experimental animals of group A, C cells were increased in size and number intrafollicularly (Fig. 3a). The degrees of immunoreaction for calcitonin, CGRP and somatostatin in C cells decreased apparently compared to control cases (Figs. 3b-d). In cases of other experimental groups, hypertrophied C cells proliferated markedly in the intrafollicular areas (Fig. 4a). Immunoreactive calcitonin, CGRP, and somatostatin conspicuously decreased in hypertrophied C cells (Figs. 4b-d). The insignificant morphological as well as immunoreactive differences in C cells of the experimental groups without group A was observed. CGA and NSE immunoreactivities in C cells of all cases of experimental animals were negative as mentioned above.

Hyperplasia and/or hypertrophy of C cells seemed to be responding to the excess of serum calcium level [5, 15]. According to the ultrastructural studies in cows [2], dogs

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Fig. 1. Experimental procedures.
Arrows indicate vitamin D₃, 40,000 IU/kg BW i.v. injection.

Table 1. The primary antisera used for this study

<table>
<thead>
<tr>
<th>Rabbit antisera against</th>
<th>Working dilution</th>
<th>Obtained from</th>
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<tbody>
<tr>
<td>Human calcitonin</td>
<td>1: 5000</td>
<td>Miles, U.S.A.</td>
</tr>
<tr>
<td>Rat CGRP</td>
<td>1: 500</td>
<td>Amersham, England</td>
</tr>
<tr>
<td>Synthetic somatostatin</td>
<td>1: 300</td>
<td>Miles, U.S.A.</td>
</tr>
<tr>
<td>Bovine chromogranin A</td>
<td>1: 100</td>
<td>Immuno Nuclear, U.S.A.</td>
</tr>
<tr>
<td>Bovine NSE</td>
<td>1: 200</td>
<td>IBL, Japan</td>
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and rats [3] with prolonged hypercalcemia induced by massive doses of vitamin D. C cells underwent hyperplasia and hypertrophy with pronounced degranulation, and contained well developed Golgi apparatus and rough-endoplasmic reticula in the cytoplasm. It is clear that C cells actively synthesize and secrete calcitonin in these conditions. C cell hyperplasia was enhanced in proportion to the duration of vitamin D₃ administration [15].

A number of immunohistochemical studies on C cells has been reported in various animals. It is well known that C cells contain calcitonin, CGRP, somatostatin, CGA, and NSE, however, the concentration of these hormones or the enzymes varies from species to species [6, 9, 10, 13, 19]. It has been reported that normal C cells of rabbits contained CGRP and somatostatin in addition to calcitonin [17, 19]. In the present study, immunoreactivity of calcitonin, CGRP, and somatostatin decreased markedly after treatment with vitamin D₃. The first change seemed to be weakening of immunoreactions connected with marked degranulation of C cells. After hypercalcemia, decrease of both calcitonin and somatostatin immunoreactivities has been observed in C cells of dogs and guinea pigs [9, 10] and immunoreactions of CGRP in addition to calcitonin decreased in rat C cells [18]. Somatostatin and CGRP in addition to calcitonin that localized in same C cells of rabbits have seemed to be the hormones regulating serum calcium level [10, 12]. Therefore, it is considered that both CGRP and somatostatin in C cells exert the synergic effect on the action of calcitonin lowering serum calcium. However, it is unclear that the relation between CGRP and somatostatin concerning their existence in C cells.

NSE, an energy providing enzyme of the glycolytic pathway, is widely distributed in various endocrine tissues [9]. CGA is an acidic protein which is stored and released with catecholamines from chromaffin cells, and is also demonstrated in neuroendocrine cells [7, 8, 14]. Both of NSE and CGA were also present in the thyroid C cells of various animals [19]. It has been reported that rabbit C cells reveal no immunoreaction to NSE [9] as well as CGA [8, 19]. In the present study, NSE and CGA immunoreactions were also not observed in normal as well as hyperplastic C cells. In hypercalcemic conditions, marked increase of NSE immunoreactivity has been observed in C cells of dogs, guinea pigs [9], and rats [18]. In hypocalcemic conditions, C cells of rabbit revealed intense immunoreactivity for both calcitonin and somatostatin, whereas no immunoreaction for NSE was noted [9]. Thus, the presence of functional proteins, such as NSE, may be connected with the functional states of C cells [9, 18]. It seems that negative immunoreactivity to NSE in C cells of rabbits depends on a low amount of the enzyme or the lack of NSE [9], and that absence of chromogranin immunoreactivity has been strongly reflected by fixation methods and/or the antisemum dilution [7, 8, 14]. The functional or physiological properties of both NSE and CGA in rabbit C cells are still unknown.

C cells secrete and/or produce certain hormones and

Figs. 2a-d. Four serial sections of the thyroid gland of a control rabbit. It is difficult to distinguish C cells from follicular epithelial cells stained with HE (a). Almost all C cells are stained densely with calcitonin (b), CGRP (c), and somatostatin (d). ×320.
Figs. 3a-d. Four serial sections of the thyroid gland of a rabbit of Group A. C cell hyperplasia (a, HE staining) and marked decrease of immunoreactive calcitonin (b), CGRP (c), and somatostatin (d) are noted. ×320.

Figs. 4a-d. Four serial sections of the thyroid gland of a rabbit of Group B. Marked C cell hyperplasia (a, HE staining) and decrease of immunoreactive calcitonin (b), CGRP (c), and somatostatin (d) are conspicuous. ×320.
proteins that may play functional significance lowering serum calcium. An immunohistochemical procedure using anti-calcitonin, anti-CGRP and anti-somatostatin antisera seemed to be useful to investigate the stimulated condition after hypercalcemia of C cells in rabbits such as in dogs, guinea pigs [11, 12] and rats [18].

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REFERENCES