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Short Communication

Differentiation behavior of pituitary cells in normal and metamorphosis-arrested larvae of the salamander *Hynobius retardatus*

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ABSTRACT When premetamorphic larvae of the salamander *Hynobius retardatus* were treated with potent goitrogens, or subjected to thyroidectomy, their metamorphosis was completely arrested. The pituitary gland of the arrested larvae consisted mostly of the hypertrophied Thyroid Stimulating Hormone (TSH) cells that are called "thyroidectomy cells". The development and dynamics of the TSH cells were studied by investigating uptake of BrdU into pituitary cell nuclei and by double-staining immunohistochemistry using anti-pituitary specific antibodies. The majority of the BrdU-positive cells expressed the TSHβ antigen, suggesting that TSH cells increased in number by their extensive proliferation in the pituitary glands of the goitrogen-treated larvae. On the other hand, double-staining immunohistochemistry showed that several prolactin (PRL) immunoreactive cells coexpressed TSHβ within single cells even in normal controls. Furthermore, pituitary cells coexpressing PRL and TSHβ increased in number in the goitrogen-treated larvae. Whereas cells coexpressing GH and TSHβ were not observed in normal controls, they appeared in the pituitary glands of the goitrogen-treated larvae. These results provide morphological evidence for considerable phenotypic plasticity in the pituitary cells of *H. retardatus*.

KEY WORDS: *double-staining immunohistochemistry, goitrogens, pituitary gland, salamander, TSH-cells*

It has been known that the pituitary cells have considerable plasticity in their phenotypic expression of own hormones. The pituitary glands of the thyroidectomized or goitrogen-treated animals contained extraordinarily large numbers of TSH-cells, which have also been called “thyroidectomy” cells (Dent, 1968). At present, however, the cellular mechanisms of the development of the hypertrophied TSH- or thyroidectomy cells are entirely unknown. Theoretically, they (1) originate from an extensive proliferation of original TSH-cells, (2) are the result of a specific differentiation from undifferentiated stem cells to TSH-cells, or (3) originate from some pituitary non-TSH-cells through a transdifferentiation of cell phenotypes, due to the lack of thyroid hormones.

In the present experiments, we examined the origin of the thyroidectomy cells, by investigating uptake of BrdU into pituitary cell nuclei and double-staining immunohistochemistry using pituitary-specific antibodies. Figure 1 shows BrdU-positive pituitary cells in the goitrogen-treated (75 days) larvae (Fig. 1B) and in the controls (Fig. 1A). The number of BrdU-positive cells in the control larvae thus far examined was the largest at 50 days after hatching. They gradually decreased in number in control pituitaries at 75 and 100 days after hatching (data not shown). In goitrogen-treated (25 days) larvae, the number of BrdU-positive pituitary cells was almost identical to the number in the controls. Fifty days after hatching, however, the number of BrdU-positive cells was much larger in the goitrogen-treated larvae than in the normal controls, suggesting extensive proliferation of pituitary cells in the goitrogen-treated larvae.

To determine what types of pituitary cells were predominantly proliferating in the pituitary glands, sections of the pituitary glands were double-stained with anti-BrdU (detected with FITC) and with anti-pituitary-specific antibodies (detected with Cy3). The specificity of the several antibodies used in this experiment was confirmed previously (Yamaguchi *et al*., 1996; Kanki and Wakahara, 1999). Nuclei of DNA-synthesizing cells stained green with FITC, but those of non-synthesizing cells did not (Fig. 1 C,D). In contrast, the cytoplasm of each pituitary cell stained red with Cy3. Combined staining with BrdU and pituitary-specific antibodies showed that many BrdU-positive cells were double-stained with anti-TSHβ anti-

Abbreviations used in this paper: ACTH, adrenocorticotropic hormone; BrdU, bromodeoxyuridine; CRH, corticotropin-releasing hormone; FCS, fetal calf serum; FITC, fluorescein isothiocyanate; FSH, follicle stimulating hormone; GH, growth hormone; GHRH, growth hormone-releasing hormone; GTH, gonadotropic hormone; LH, luteinizing hormone; MSH, melanophore stimulating hormone; PRL, prolactin; TSH, thyroid stimulating hormone.

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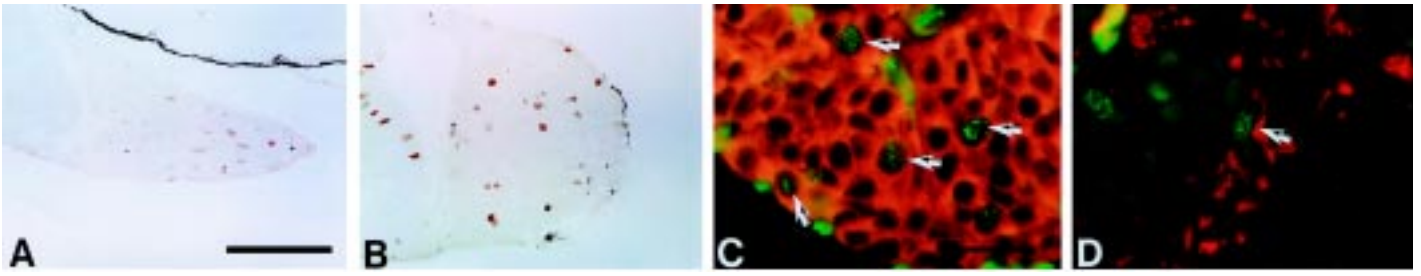

Fig. 1. Immunohistochemical detection of BrdU in pituitary cells of *Hynobius retardatus*. (A) *Normal control and* **(B)** *goitrogen-treated larva. Much more BrdU-positive cells (detected by peroxidase activity) were observed in the pituitary gland of the goitrogen-treated larva than in that of the control. (C,D) Double-staining immunohistochemistry of BrdU (detected by FITC, green fluorescence) and pituitary-specific antibodies (detected by Cy3, red) in goitrogen-treated larvae.* **(C)** *Double-staining for BrdU and TSHβ. Several cells are stained with BrdU in their nuclei and with TSHβ in their cytoplasm (arrows), showing that many TSH immunoreactive cells synthesize DNA.* **(D)** *Double-staining for BrdU and PRL. A BrdU-positive cell simultaneously expressed PRL antigens (arrow). Bars: 100 μm (A,B); 20 μm (C,D).*

body (Fig. 1C). Similarly, some BrdU-positive cells were double-stained with anti-PRL (Fig. 1D), anti-ACTH, or anti-GH antibodies. In contrast, BrdU uptake was hardly observed in GTH-cells, suggesting no proliferation of GTH-cells at this stage. Figure 2 shows the proportions of double-positive cells with BrdU and pituitary-specific peptide antibodies relative to the total number of BrdU-positive cells in the goitrogen-treated larvae. Approximately 1000 double-positive cells from the goitrogen-treated (50 days) larvae were observed under a fluorescence microscope, and their expressed antigens were analyzed. Approximately 90% of BrdU-positive cells expressed TSHβ antigens. About 10%, 5%, and 5% of the BrdU-positive cells expressed PRL, ACTH, and GH, respectively. Because some pituitary cells simultaneously expressed different antigens within single cells (described later), the sum of the percentages of each cell type totaled more than 100%. It is thus concluded that the development of an extraordinarily large number of TSH-cells in the goitrogen-treated larvae can be attributed, at least in part, to extensive proliferation of original TSH-cells. In this respect, Miranda *et al*. (1995) have shown that, in *Bufo arenarum*, both TSH and PRL cell populations and cell volumes increased in goitrogen-treated tadpoles compared with normal larvae at the same stage. Similarly, in *H. retardatus*, extensive proliferation of TSH-cells and a certain degree of proliferation of PRL-, ACTH- and GH-cells, but not of GTH-cells, was induced in the pituitary glands of goitrogen-treated larvae. *In situ* hybridization has clearly shown that almost all pituitary cells in goitrogen-treated or in thyroidectomized larvae express TSHβ mRNA (Kanki and Wakahara, 2000). Thus, it seems reasonable to assume that in *H. retardatus* a chronic shortage of thyroid hormones caused by the goitrogen-treatment stimulates TSH-releasing activity from the hypothalamus (probably CRH in amphibians, Denver and Licht, 1989; Gancedo *et al*., 1992) by a strong negative feedback system and results in the hypertrophy of TSH-cells by stimulating transcription of TSH-messengers.

To examine the origin of the thyroidectomy cells, double-staining immunohistochemistry was examined using guinea pig anti-TSHβ antibody and rabbit antibodies against PRL, ACTH, GH, and GTH as primary antibodies, and anti-guinea pig IgG conjugated with FITC (for TSHβ) and anti-rabbit IgG conjugated with Cy3 (for PRL, ACTH, GH, and GTH) as secondary antibodies (Fig. 3). The double-staining immunohistochemistry demonstrated that a certain proportion of the pituitary cells expressed both TSHβ and PRL simultaneously even in the normal controls, suggesting that both antigens were coexpressed within single cells. In contrast, coexpression of TSHβ and either ACTH, GH, or GTH was not observed in the pituitary glands of the normal controls. Cells that expressed TSHβ and PRL simultaneously increased in number in the goitrogen-treated larvae (Fig. 3C). Furthermore, cells expressing TSHβ and GH simultaneously were detected in the goitrogen-treated larvae (Fig. 3F), whereas such cells were seldom observed in the controls. Coexpression of TSHβ and either ACTH or GTH was not observed at all in either the goitrogen-treated larvae or in the normal controls. Because FSH and LH are similar glycoprotein hormones composed of a common α-subunit and specific β-subunits, and ACTH and αMSH contain common amino acid sequences, it seems possible that almost all gonadotrophs synthesize and secrete both FSH and LH (Gracia-Navarro and Licht, 1987; Tanaka *et al*., 1990) and that some secretory granules contain ACTH and αMSH (Tanaka and Kurosumi, 1986). In *H. retardatus*, however, it was demonstrated that some PRL-cells simultaneously coexpressed TSHβ within single cells in normal controls, and that the cells coexpressing TSHβ and PRL increased in number in the goitrogen-treated larvae (Fig. 3C). Furthermore, cells that coexpressed

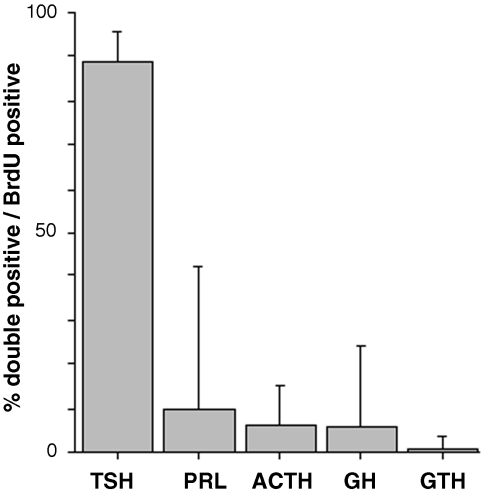

Fig. 2. Proportions of TSH-, PRL-, ACTH-, GH- and GTH-cells relative to the total number of BrdU-positive cells in goitrogen-treated larvae. *Approximately 90% of BrdU-positive cells expressed TSHβ antigens. About 10%, 5%, and 5% of the BrdU-positive cells expressed PRL, ACTH, and GH, respectively. Because some pituitary cells simultaneously expressed different pituitary-specific antigens within single cells, the sum of the percentages of each cell type is over 100%.*

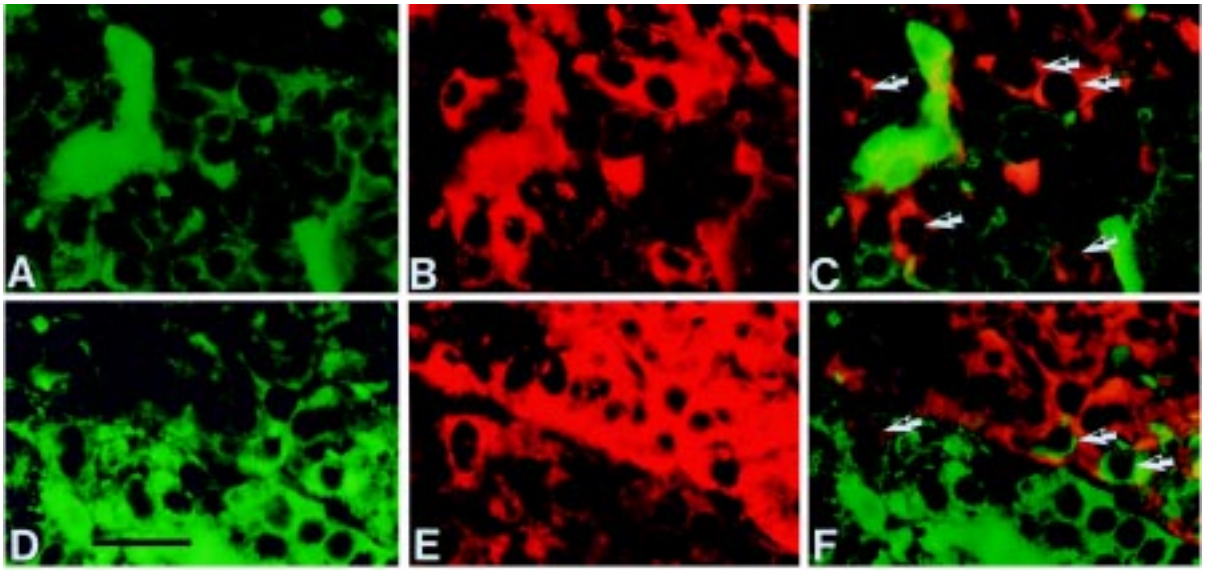

Fig. 3. Double-staining immunohistochemistry of pituitary glands. *(A,B,C) Double-staining with TSHβ (FITC) and PRL (Cy3). The section was observed with different excitation filters for:* **(A)** *FITC (TSHβ),* **(B)** *Cy3 (PRL), and* **(C)** *merge (double-stained with TSHβ and PRL). (D,E,F) Double-staining with TSHβ (FITC) and GH (Cy3). The section was observed with different excitation filters for:* **(D)** *FITC (TSHβ),* **(E)** *Cy3 (GH), and* **(F)** *merge (double-stained with TSHβ and GH). Double-stained cells (arrows) emitted orange fluorescence mixed with FITC (green) and Cy3 (red). Bar, 20 μm.*

TSHβ and GH appeared in the pituitary gland of the goitrogen-treated larvae (Fig. 3F). These observations lead us to conclude that some pituitary cells have an ability to produce several kinds of peptide hormones regardless of their differentiation, or, in other words, they show a plasticity of phenotypic expression as discussed below.

In *in vivo* and *in vitro* experiments in mammals, transdifferentiation of pituitary cells between PRL- and GH-cells (Kineman *et al.*, 1992; Horvath and Schally, 1994; Goda *et al*., 1998), and GH- and TSH-cells (Vidal *et al*., 2000) has been demonstrated under various experimental conditions. These observations support the concept that pituitary cells are not irreversibly committed to the production of one single hormone and that their phenotype can change in response to hypothalamic regulation. Recent progress in research on signaling mechanisms in pituitary morphogenesis and cell fate differentiation have shown that cells expressing Pit-1, the pituitary-specific transcription factor, can mature into GH-, PRL-, or TSH-cells (Asa and Ezzat, 1999; Rhodes *et al*., 1994; Sanno *et al*., 1996). These studies suggest the presence of common stem cells for GH-, PRL-, and TSH-cells in developing pituitary glands. This is consistent with our results reported here that PRL-cells coexpress TSHβ even in normal controls, and that some GH-cells come to express TSHβ in goitrogen-treated larvae (Fig. 3F). On the one hand, it is possible that TSH-cells in the goitrogen-treated larvae derived directly from differentiated pituitary non-TSH-cells through transdifferentiation. It seems premature at present to decide which is the case in goitrogen-treated *H. retardatus*: the specific differentiation from stem cells to TSH-cells or the transdifferentiation of differentiated pituitary non-TSH-cells to TSH-cells. Even non-pituitary glands such as parotid and salivary glands have been reported to be able to produce LH (Tresguerres *et al*., 1999a) and GH (Tresguerres *et al*., 1999b) after stimulation with LH-releasing hormone (LHRH) and GHRH, respectively. Taken together, these findings suggest that hypothalamic neuroendocrine signals have a strong ability to promote differentiation of pituitary cells or to produce different types of pituitary hormones in various tissues and cells. This is in accordance with a recent study showing an extrapituitary expression of the PRL gene in goldfish, African clawed frog, and mouse (Imaoka *et al*., 2000).

Experimental Procedures

Animals

Fertilized eggs of *Hynobius retardatus* were collected during the breeding season from ponds in the vicinity of Sapporo (Iwasaki and Wakahara, 1999). Newly hatched larvae were reared at room temperature either in a mixed solution of 0.02% thiourea and 0.04% sodium perchlorate (TU-SPC) to arrest their metamorphosis (Wakahara and Yamaguchi, 1996) (experimentals) or in goitrogen-free medium (controls). Metamorphosis of the larvae was arrested at stage 63 (full-grown larval stage, just prior to metamorphosis) by the goitrogen treatment. The larvae were fed with live *Tubifex* or frozen red worms.

BrdU uptake

Three larvae from each goitrogen-treated group (25, 50, 75, and 100 days after hatching) and their controls were injected with BrdU 24 h before fixation (Kanki and Wakahara, 1999). Paraffin-embedded pituitary glands were prepared for histology. Five-micrometer sections were processed for immunohistochemistry using mouse anti-BrdU as a primary antibody and peroxidase-conjugated anti-mouse IgG as a secondary antibody. Peroxidase activity was detected with diaminobenzidine (DAB) as a substrate. To perform double-staining immunohistochemistry of BrdU and pituitary-specific antibodies, mouse anti-BrdU and rabbit anti-pituitary peptides were used as primary antibodies. FITC-conjugated anti-mouse IgG was used as a secondary antibody for the detection of BrdU, and Cy3-conjugated anti-rabbit IgG was used for the detection of pituitary-specific antibodies.

Immunohistochemistry

For double-staining immunohistochemistry, pituitary glands were fixed with 4% paraformaldehyde overnight. Sections were blocked for 30 min with 10% FCS-PBS at 4°C. Guinea pig anti-human TSHβ was used as the primary antibody. After washing, FITC-conjugated anti-guinea pig IgG was applied to the preparations as a secondary antibody. The same preparations were

treated with either rabbit anti-human PRL, anti-human ACTH, anti-human GH, or with mixed antibodies of anti-bullfrog FSHβ and LHβ as primary antibodies, and then treated with anti-rabbit IgG conjugated with Cy3 as a secondary antibody under conditions similar to those described above. The sections were observed with excitation filters for FITC (TSHβ), for Cy3 (pituitary-specific antibodies other than TSHβ), and for FITC and Cy3 (double-staining).

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