SOMATOSTATIN-IMMUNOREACTIVE C-CELLS IN PRIMARY CULTURE OF FETAL, NEONATAL AND YOUNG RAT THYROID GLANDS

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ABSTRACT

Developmental change in somatostatin expression in calcitonin cells (C-cells) was studied in the primary cultures prepared from fetal and baby rat thyroid glands. Enzymatically dispersed thyroid glands were cultured for 48 h in Dulbecco's modified Eagle's medium supplemented with 5% fetal bovine serum. Localization of calcitonin- and somatostatin-immunoreactivities in these cultures was examined by double fluorescence staining. Almost all somatostatin-immunoreactive cells exhibited calcitonin-immunoreactivity. The frequency of somatostatin-immunoreactive cells was more than 80% of all C-cells in the cultures prepared from rats at 14th and 15th day of gestation. In the cultures from rats at 16th-21st day of gestation, approximately 60% of C-cells exhibited also somatostatin-immunoreactivity. The ratio of somatostatin-immunoreactive cells to calcitonin-immunoreactive cells significantly decreased to 8% and 1% in the cultures from 10-day-old and 28-day-old rats, respectively.

Somatostatin (somatotropin release-inhibiting factor) is a tetradecapeptide having a very conservative amino acid sequence. Somatostatin was first found in the hypothalamus (3), and has been demonstrated to localize in other parts of the central and peripheral nervous tissue. Somatostatin-immunoreactivities have also been found in the endocrine organs such as pancreatic islets and thyroid glands (21).

In the thyroid glands of rats and rabbits, somatostatin was shown to be stored within the same secretory vesicles with calcitonin in calcitonin cells (C-cells) (23, 24). Although there are several reports that exogenous somatostatin inhibits secretion of calcitonin (7, 11, 12, 16) or thyroid hormone (1, 17), the physiological role of somatostatin stored in C-cells is

still unknown.

The ratio of somatostatin-immunoreactive cells to calcitonin-immunoreactive cells depends highly upon both the species (13, 14) and ages (2, 15) of animals. According to the immunocytochemical studies by Larsson (15), nearly 90% of the C-cells were also somatostatin-immunoreactive in neonatal rat pups up to day 6, and the ratio markedly decreased to 25% at day 8, and to less than 1% in adult. It is still unknown why the pronounced change in somatostatin contents occurs in the thyroid C-cells between day 6 and day 8 in their neonatal life.

Although C-cell lines which produce and secrete somatostatin were established from medullary thyroid carcinoma (9, 18, 25), no information has been available on somatostatin production in the primary culture of C-cells. We previously reported that calci-

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tonin- and calcitonin gene-related peptide (CGRP)-immunoreactive cells in the primary cultures of normal C-cells prepared from fetal rat thyroid glands (19). In the present study, expression of immunoreactive somatostatin was detected in the primary culture of C-cells by double fluorescence staining.

MATERIALS AND METHODS

Animals

Wistar-Imamichi rats (Imamichi Institute for Animal Reproduction) were kept on a cycle of 14 h light (0600–2000) and 10 h darkness, and were fed unlimited diet (Oriental Yeast) and water. A female rat at proestrus was caged with a male between 1800–0900, and the day on which sperm were found in the vaginal smear was designated as day 0 of pregnancy. The fetus at gestational day *X* was designated as GX fetus. Fetal (G14–G21), 10-day-old, and 28-day-old rats were used for the experiments.

Cell Culture

Primary cultures of rat thyroid C-cells were prepared as described previously (19) with modifications. Thyroid glands of the rats were taken under a dissecting microscope and rinsed with Hanks' balanced salt solution (HBSS). Enzymatic dissociation was carried out by adding 1 ml of HBSS containing 1 mg/ml collagenase (Worthington, CLS II) and 1,000 units/ml Dispase (Godo Shusei). After 1 h of incubation at 37°C, the glands were mechanically dispersed by pipetting through a silicon-coated capillary pipette, and were filtrated through a stainless sieve (150 μ m-opening). The filtrated cells were washed twice by gentle centrifugation (100 g, 5 min) with 2 ml of Dulbecco's modified Eagle's medium (Gibco, No. 430-1600) supplemented with heat-inactivated serum of fetal bovine (5%; Gibco, No. 230-6140), penicillin and streptomycin (50 units/ml and 50 μ g/ml respectively; Gibco, No. 600-5070). Then the cells were resuspended in an appropriate volume of the same culture medium, and were distributed equally onto fibronectin-coated glass coverslips (50 μ l cell suspension/coverslip) in a 24-well multidish (Falcon). The cell density was controlled to generate approximately 200 (G14 fetuses) or 500 (G15 fetuses–

28-day-old rats) C-cells/well after 48 h of culture. After 2 h of incubation at 37°C in a humidified atmosphere of 5% CO₂ in air, additional medium (450 μ l) was gently added to each well. The cells were cultured under the same conditions for 48 h.

The culture medium stored at 4°C was used within a month. Extirpation of the thyroid glands was started at 1300.

Immunocytochemistry

Calcitonin and somatostatin were detected in the same specimen by double fluorescence staining as described below. The cultures were fixed in 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.4) for 30 min, and washed with PBS. The fixed cells were treated with 0.25% Triton X-100 in PBS containing 10 mg/ml bovine serum albumin (BSA, Sigma Fraction V). After 30 min of incubation with 10 mg/ml BSA in PBS, the permeabilized cells were incubated for 1 h with a mixture of primary antisera; goat antiserum to human calcitonin (Cambridge Medical Diagnostics) and rabbit antiserum to somatostatin (ICN ImmunoBiologicals). The specimens were rinsed with PBS, and incubated for 30 min with rhodamine-labeled swine antiserum to goat IgG (Tago). After rinsing with PBS, they were incubated for 30 min with fluorescein-labeled sheep antiserum to rabbit IgG (Cappel). All the antisera were used after diluting to 1/200 with PBS containing 10 mg/ml BSA. The specimens were rinsed with PBS, embedded in glycerin-PBS (1:1, v/v), and examined under a fluorescence microscope. As negative controls, 1) normal sera of respective animals were substituted for either of the primary antisera, 2) the mixture of the primary antisera was incubated with excess somatostatin (100 μ g/ml) for 1 h before the experiments. Immunocytochemical procedures described above were carried out at room temperature.

Evaluation of the Ratio of Somatostatin-immunoreactive Cells to Calcitonin-immunoreactive Cells

At first, the photomicrographs of the double fluorescence-labeled specimens were taken at the same field with respective excitation, and

Table 1 *Number of Calcitonin- and Somatostatin-immunoreactive Cells in the Cultures Derived from the Thyroid*

Age		Calcitonin-immunoreactive cells/thyroid (a)	Somatostatin-immunoreactive cells/thyroid (b)	b/a (%)
Gestational				
Day	14	51± 3	41± 4	81.0±3.3
	15	99± 17	83± 16	83.5±8.3
	16	1,030± 75	656± 67	63.5±2.1
	17	1,275± 97	774±102	60.5±4.0
	18	1,293±111	793± 73	61.5±5.1
	19	1,314±113	676± 70	51.8±6.6
	20	1,359±111	790± 58	58.3±2.4
	21	2,059±230	1,193±164	58.0±5.0
Postnatal				
Day	10	1,434±177	123± 57	8.3±3.1
	28	2,987±616	17± 10	0.8±0.4

The values are means±SD of at least four independent experiments. The number of somatostatin-immunoreactive cells which did not exhibit calcitonin-immunoreactivity was negligible in all age groups.

it was examined whether all the somatostatin-immunoreactive cells were also calcitonin-immunoreactive. Secondly all the calcitonin- and somatostatin-immunoreactive cells found on each coverslip were counted and the ratio of somatostatin-immunoreactive cells to calcitonin-immunoreactive cells was calculated. At least three coverslips were used for counting in each experiment. The results were expressed as mean value±SD of four or more independent experiments.

RESULTS AND DISCUSSION

In our culture system, the number of C-cells derived from a single thyroid gland increased with the age except the experiments using 10-day-old rats (Table 1). This increase in the number of C-cells could result partly from an increase in the number of C-cells and/or C-cell precursor cells which had been in the thyroid glands used for cell culture. According to a previous study by Garel *et al.* (10), the number of C-cells in the thyroid glands increased with the age during fetal and postnatal (at least up to 15-days) period in rats. At present, we have no experimental result to explain the unexpectedly small number of C-cells found in the cultured thyroid cells of 10-day-old rats.

Factors such as the adhesiveness to the substratum, the rate of calcitonin production and secretion, and the proliferation rate of C-cells might possibly be affected during the functional adaptation from the fetal life to the postnatal life. It has been observed that serum calcitonin level reaches a plateau at the age of day 3 through day 10 and it decreases toward the adult level till day 21 in Wistar-Imamichi rats (8).

Preliminary experiments by photomicrography revealed that almost all somatostatin-immunoreactive cells co-expressed calcitonin-immunoreactivity in the cultures of every age group studied. The C-cells exhibiting somatostatin-immunoreactivity alone were negligible in the total C-cells. In the experiments mentioned below, therefore, the ratio was calculated from the numbers of calcitonin- and somatostatin-immunoreactive cells counted separately under a fluorescence microscope as described in Materials and Methods.

More than 80% of the calcitonin-immunoreactive cells were somatostatin-immunoreactive in the cultures prepared from G14 (Table 1 and Fig. 1, A and B) and G15 (Table 1, and Fig. 2, A and B) fetuses. The ratio decreased to 64% in the cultures from G16 fetuses, and the ratio was rather constant in the cultures

Figs. 1 and 2 Immunofluorescence photomicrographs showing somatostatin (A)- and calcitonin (B)-immunoreactivities of the same field. The primary cultures were prepared from the rat fetuses at 14th (Fig. 1) and 15th (Fig. 2) day of gestation, and were subjected to immunocytochemical procedures after 48 h. $\times 160$

from G16–G21 fetuses (Table 1, and Fig. 3, A and B). In the cultures prepared from 10-day-old and 28-day-old rats, the ratio of somatostatin-immunoreactive cells to calcitonin-immunoreactive cells significantly decreased to 8% and 1%, respectively (Table 1, and Fig. 4, A and B).

When the antiserum to somatostatin was omitted or preabsorbed with synthetic somatostatin, somatostatin-immunoreactivity was not observed, whereas calcitonin-immunoreactivity was not affected. When the antiserum to calcitonin was omitted, somatostatin-immunoreactivity alone was observed. These results show that each antiserum was specific

enough for this double labeling system.

C-cells were known to produce several biologically active peptides: calcitonin, CGRP, katecalcin, calcitonin carboxy terminal adjacent peptide (4), somatostatin (21), and in C-cell lines, neurotensin (25). Gastrin- or cholecystokinin-like (15) and helodermin-like (22) immunoreactivities were also demonstrated in rat and human C-cells respectively, but the chemical entities of these immunoreactants have not been determined. Although there have been many immunohistochemical studies on the localization of these peptides at both the light and electron microscopic level, their physiological roles have not been fully

Figs. 3 and 4 Immunofluorescence photomicrographs showing somatostatin (A)- and calcitonin (B)-immunoreactivities of the same field. The primary cultures were prepared from rat fetuses at 20th day of gestation (Fig. 3) and 10-day-old rats (Fig. 4), and were subjected to immunocytochemical procedures after 48 h. $\times 160$

elucidated.

We previously reported that somatostatin-immunoreactive cells appeared in cultures derived from rat fetuses at G14 or later stages (20). But it was not clear whether or not calcitonin- and somatostatin-immunoreactivities were expressed in the same cells. According to the immunohistochemical studies by Kameda *et al.* (13), in the young rats, somatostatin is contained in the same C-cells that contain calcitonin, and there are some C-cells showing much stronger immunoreaction for somatostatin than for calcitonin. For the rat fetuses, however, the coexistence of calcitonin and somatostatin in the thyroid C-cells has not

been examined. Our present study revealed that almost all the somatostatin-immunoreactive cells in the culture were regarded as a subpopulation of C-cells. There are a few cells (less than 1% of total cells exhibiting calcitonin- or somatostatin-immunoreactivity) showing somatostatin-immunoreactivity alone in the cultures from the rats of every age. It is unclear whether such somatostatin-immunoreactive cells contain a trace amount of calcitonin below the limit of immunofluorescence detection.

In the cultured C-cells described here, CGRP-immunoreactive cells were also found as a subpopulation of C-cells. Although the

ratios of somatostatin-immunoreactive cells to calcitonin-immunoreactive cells were variable according to the age of rats used for the experiments, the ratios of CGRP-immunoreactive cells to calcitonin-immunoreactive cells were found to be rather constant throughout the ages. The ratio of CGRP-immunoreactive cells to calcitonin-immunoreactive cells was approximately 80% in the cultures from G16-G19 fetuses and was more than 90% in the cultures from 10-day-old and 28-day-old rats (Nishiyama and Fujii, unpublished data).

It was reported in rats that the ratio of somatostatin-immunoreactive cells to calcitonin-immunoreactive cells rapidly decreased in the neonatal period (2, 15). Cultured C-cells, in the present study, reflected in part the states of C-cells *in vivo*: C-cells expressed somatostatin-immunoreactivity at high frequency in the culture of G21 fetuses, and at quite low frequency in the cultures of 10-day-old and 28-day-old rats. According to our preliminary experiments, enrichment of FBS in the culture medium up to 15% did not alter the ratio of somatostatin-immunoreactive cells to calcitonin-immunoreactive cells at least up to 72 h of incubation. In an attempt to manipulate somatostatin production *in vitro*, C-cells were cultured in the medium supplemented with fetal or adult rat serum. But the ratio of somatostatin-immunoreactive cells to calcitonin-immunoreactive cells did not change up to 48 h of incubation (data not shown). In C-cell lines established from medullary thyroid carcinoma, glucocorticoids have been shown to modulate differentially calcitonin gene expression at both the transcriptional and splicing level (5, 18, 25, 26). Glucocorticoids also inhibit somatostatin production in the TT cell lines (6). Studies on the artificial alteration in somatostatin expression *in vitro* were now under way in our laboratory by using various hormones including glucocorticoids as well as the sera from rats of various ages. Further investigations are obviously necessary to establish a model system for studying the age-related peptide hormone expression.

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