Developmental Change in Calcitonin Secretory Capacity of Fetal Rat Thyroid C-Cells

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Abstract—Developmental changes in calcitonin (CT) secretory capacity of C-cells were studied by using primary cultures prepared from the thyroid glands of rat fetuses at 16th, 18th and 20th day of gestation and the thyroid glands of 28-day-old rats. Both CT content of C-cells and high Ca2+-stimulated CT secretion increased with the age of the rats. The ratio of secreted CT to the CT content of C-cells also increased according to the age. These results demonstrated the functional development of C-cells during the fetal period.

The major role of the thyroid C-cell is to secrete calcitonin (CT) in response to elevated Ca2+ levels in the blood. The ontogenetic aspects of the development of the C-cell has been studied with immunohistochemical techniques in various animals (1-3) including rats (4-7). According to the quantitative studies by Garel et al. (4), both the CT content of the thyroid glands and plasma CT concentration increase with age in the rat fetuses. However, the developmental change in CT secretory capacity of C-cells in response to high Ca2+ has not been well studied. We previously succeeded in establishing culture systems for rat fetal thyroid C-cells (5, 6). In the present study, the primary cultures of C-cells were prepared from the thyroid glands of fetal and young rats and were examined for their capacity to secrete CT in response to an increase in extracellular Ca2+ concentration.

Wistar-Imamichi rats (Imamichi Institute for Animal Reproduction, Saitama) were used for the experiments. The day on which sperm were observed in the vaginal smear was designated as day 0 of pregnancy. When the ontogenetic appearance of C-cells was examined by using the immunoreaction for CT in the culture of fetal thyroid glands, a stable number of C-cells was found at gestational day 16 or later stages (6). In this study, therefore, the primary cultures of the thyroid glands were prepared from rat fetuses at the 16th (G-16), 18th (G-18) and 20th (G-20) day of gestation and from 28-day-old rats. Ten thyroid glands were pooled and enzymatically dispersed as previously described (5). The dispersed cells were suspended in Dulbecco’s modified Eagle’s medium supplemented with heat-inactivated fetal bovine serum (5%), penicillin (50 units/ml) and streptomycin (50 μg/ml). The cell suspension was equally distributed onto 10 (G-16), 15 (G-18 and G-20) or 45 (28-day-old) glass coverslips (14 mm-diameter) placed in 24-well multidishes (Falcon, Becton Dickinson, Franklin Lakes, NJ). The cultures were maintained at 37°C in a humidified atmosphere of 5% CO2 in air and used for the experiments described below after 48 hr.

For determining the CT content of C-cells, the cultures were washed three times with 25 mM N-[2-hydroxyethyl]piperazine-N’-[2-ethanesulfonic acid] (HEPES)-buffered Hanks’ balanced salt solution (HBSS, pH 7.3) containing 1 mM CaCl2. To each well was added the extraction medium (1 ml) containing 15 mM NaCl, 1 mM HCl and 1 mM phenylmethylsulfonyl fluoride (PMSF). The cells were frozen at −40°C and stored until assayed. The frozen cells were thawed and homogenized with a ultrasonic cell disruptor.

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(Microson MS-50, Heat Systems-Ultrasonics Inc., Farmingdale, NY). The homogenate was transferred from each well to a centrifuge tube and centrifuged at 4°C (2,000×g, 15 min). The resultant supernatant was assayed for CT.

To determine CT secretory capacity of C-cells, the cultures were washed with HHBSS as described above and then incubated at 37°C in humidified air with HHBSS (0.3 ml) containing 1 mM or 3 mM CaCl₂. After 30 or 60 min, the medium was collected and centrifuged at room temperature (500×g, 10 min). The supernatant was stored at -40°C until assayed for CT.

The concentration of immunoreactive CT was determined by using a commercially available radioimmunoassay (RIA) kit for human CT according to the supplier's recommendation.

In the parallel experiments to determine the cellular CT contents and CT secretory capacity of C-cells, C-cells were detected by indirect immunofluorescence techniques as previously described (5). The number of C-cells was counted under a fluorescence microscope at ×200 magnification, and the cellular CT contents and the amount of secreted CT per C-cell were calculated.

The medium, fetal bovine serum and antibiotics used for cell culture were purchased from Gibco Lab. (Grand Island, NY). HEPES and PMSF were obtained from Sigma Chemical Co. (St. Louis, MO). The RIA kit was a product of Eiken Chemical Co. (Tokyo).

Figure 1 shows the typical phase-contrast (A) and CT-immunofluorescence (B) images of the cultured cells prepared from the thyroid glands of G-20 rat fetuses. Both monolayer and multilayer arrangements were observed in the confluent cell sheet. The clusters consisting of several C-cells were frequently observed in the cultures (Fig. 1B). Practically the same phase-contrast and immunofluorescence images were observed in the experiments using G-16, G-18 and 28-day-old rats except that the intensity of the specific fluorescence was found to increase with the age of the rats.

Because the primary cultures were prepared from the whole thyroid glands of rats at various developmental stages, both the ratios of C-cells to the total cells and CT contents of C-cells were expected to be variable according to the age of the rats. Therefore, we determined for each culture well the number of C-cells and total amount of cellular CT as well as the concentration of secreted CT in the medium.

After 48 hr of culture, C-cell densities in

Fig. 1. Phase-contrast (A) and calcitonin immunofluorescence (B) images of cultured cells prepared from thyroid glands of rat fetuses at 20th day of gestation. The cells were cultured for 48 hr. (A) ×150, (B) ×200.
| Age of rats     | CT Content (fg/C-cell) (a) | Incubation period (min) | 1 mM Ca²⁺ | 3 mM Ca²⁺ |
|----------------|--------------------------|-------------------------|-----------|-----------|
|                |                          |                         | Secreted CT (fg/C-cell) (b) | [b/a (%)] | Secreted CT (fg/C-cell) (c) | [c/a (%)] |
| Gestational day|                          |                         |           |           |
| 16             | 284.8±13.5               | 30                      | <5.0      | [<1.8]    | <5.0                       | [<1.8]   |
|                |                          | 60                      | <5.0      | [<1.8]    | <5.0                       | [<1.8]   |
|                |                          | P<0.001                 |           |           |
| 18             | 497.0±9.3                | 30                      | <7.5      | [<1.5]    | 11.6±4.1                   | [2.3±0.8]|
|                |                          | 60                      | <7.5      | [<1.5]    | 18.3±8.1                   | [3.7±1.6]|
|                |                          | P<0.001                 |           |           |
| 20             | 734.3±53.6               | 30                      | <7.0      | [<1.0]    | 59.8±11.2**                | [8.1±1.5**]|
|                |                          | 60                      | <7.0      | [<1.0]    | 115.6±15.1**               | [15.7±2.1**]|
|                |                          | P<0.001                 |           |           |
| Postnatal day  |                          |                         |           |           |
| 28             | 1319.3±44.9              | 30                      | 14.2±3.1  | [1.1±0.2] | 84.3±22.4***,**           | [6.4±1.7***,**] |
|                |                          | 60                      | 19.0±7.5  | [1.4±0.6] | 156.6±17.1**,**,**        | [11.9±1.3**,**] |

(a): The number of C-cells and the amount of cellular calcitonin (CT) were determined in parallel experiments, and the CT content of C-cells was calculated. Means±S.E.M. (N=4). (b) and (c): The cultured cells were incubated in the medium containing 1 mM (b) or 3 mM (c) Ca²⁺ for 30 and 60 min. The amounts of secreted CT per C-cell were indicated as means±S.E.M. (N=4). (b/a) and (c/a): The percentages of secreted CT to the CT content of C-cells were calculated and were indicated as means±S.E.M. (N=4) in the square brackets. **: P<0.01 vs. the corresponding values of gestational day 18; †: P<0.05 vs. the corresponding values of gestational day 20; **: P<0.01 vs. basal CT secretion in the medium containing 1 mM Ca²⁺ by Student's t-test.
the cultures prepared from G-16, G-18, G-20 and 28-day-old rats were estimated to be 1203±98, 798±97, 852±51 and 678±80 C-cells/well, respectively (mean±S.E.M., N=8). As was expected from the immunocytochemical observations, the CT content of the cultured C-cells increased with the age of the rats (Table 1). The CT contents (approx. 0.3-1.3 pg/C-cell) are in the same range as those reported by Garel et al. (0.5-1.5 pg/C-cell) (4).

In the cultures of G-16, G-18 and G-20 fetal thyroid glands, CT secretion was not measurable up to 60 min of incubation in the medium containing 1 mM Ca²⁺ (basal CT secretion). In the cultured C-cells prepared from 28-day-old rats, basal CT secretion reached to a detectable level within 30 min of incubation (Table 1). An increase of extracellular Ca²⁺ concentration from 1 to 3 mM failed to induce measurable CT secretion within 60 min of incubation in the cultures prepared from G-16 fetuses, whereas it evoked CT secretion from the cultured C-cells of G-18, G-20 and 28-day-old rats (Table 1). The amount of secreted CT increased in proportion to the incubation period. The CT secretion induced by 3 mM Ca²⁺ increased according to the age of the rats. The ratio of secreted CT to the CT content also increased with age. In the cultures of G-18 fetal thyroid glands, secreted CT in 60 min of incubation was only 3.7% of the cellular CT, and the ratio increased to 15.7% and 11.9% for the cultures from G-20 and 28-day-old rats, respectively (Table 1).

During ontogeny of the C-cells, high Ca²⁺-induced CT secretion and the ratio of secreted CT to the CT content increased in different patterns. These results suggest that the age-dependent increase in CT secretory capacity of C-cells results from at least two types of changes in C-cells: one is an increase in CT content, and the other is an unknown change(s) in the mechanism concerning CT secretion. The possible candidates of the latter unknown change(s) are the changes in 1) the rate of CT biosynthesis, 2) the mechanism concerning the Ca²⁺ signal transduction system including protein kinase (8-10) A and C pathways and Ca channels (11-14), and 3) the secretory machinery itself. Further experiments are obviously required for concluding what change is responsible for the increase in CT secretion rate during the fetal period.

In the present study, we clearly demonstrated an increase in CT secretory capacity of cultured C-cells during the fetal period in response to a Ca²⁺ challenge. CT secretory capacity of G-20 fetal C-cells developed to the same level as that of 28-day-old rats in terms of the ratio of secreted CT to the total CT content. These results suggest that CT secretion in response to high Ca²⁺ plays crucial roles at and/or after birth. It is reasonable that the CT secretory function of C-cells would be well-developed before birth because one of the physiological roles of CT is to minimize the hypercalcemia during and after feeding (15).

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