An Immunocytochemical Study on the Development and Growth of C-cells in the Rat Thyroid

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Abstract This study was performed to investigate the developmental change of the C-cells in the thyroid glands of the rats. The animal groups used in this study included 17.5-, 18.5-, 19.5-, 20.5-, 21.5-day-old rat fetuses and 3-, 7-, 15-day-old neonatal rats and adult rats weighing about 200 gm. The specimens were fixed with Bouin’s fluid and embedded in paraffin. Transverse serial sections of the thyroid were cut at 5 μm from the superior to the inferior pole and eleven representative sections per thyroid lobe were selected with same intervals for immunohistochemical staining. The sections were subjected to the immunoperoxidase staining using anti-synthetic human calcitonin antibody for the detection of C-cells and counterstained with hematoxylin. Then, they were observed under the light microscope and the results were as follows:

1. Immunoreactive C-cells were first observed in the thyroid from the 20.5-day-old fetuses, but in only one and in all thyroids from 21.5-day-old fetuses.
2. The area of the thyroid gland containing C-cells, which was the posteroentral area of the lobe in 21.5-day-old fetuses, increased with aging. It occupied almost entire thyroid except for a small peripheral zone and the polar regions in the adult rats.
3. The numbers of C-cells per unit area (in this study, defined as 227 μm × 227 μm) of the thyroid from 21.5-day-old fetuses, 3-, 7-, 15-day-old rats and adult rats were 17.8 ± 4.8, 16.2 ± 4.9, 20.8 ± 4.4, 22.6 ± 6.1, 54.7 ± 9.3, respectively.
4. The ratios of intrafollicular C-cells to total C-cells were 18.3%, 20.0%, 29.5%, 30.3% in the specimens from 3-, 7-, 15-day-old rats and adult rats, respectively.
5. The ratio of C-cells which appeared singly to total C-cells was decreased with aging from 88.8% in the 21.5-day-old fetus thyroids to 33.6% in the adult thyroids.
6. C-cells in the adult rat thyroids were polymorphic in shape; round, ovoid, triangular and spindle-shaped. They were relatively uniform in size with the diameter of 13.2±2.3 μm. C-cells in the fetal thyroid were variable in size ranging from 5.6 μm to 11.3 μm in diameter and had less cytoplasm than that of adult rat.

Key words: Immunoreactive C-cell, Thyroid gland, Embryonic development and growth

INTRODUCTION

Since Baber (1876) first described the parafollicular cells in the dog thyroids, the parafollicular cells in the mammalian thyroid glands have been studied intensively by many investigators. The function of the parafollicular cells has been thought to be different from that of the follicular cells. In 1966, Pearse showed the fact that the parafollicular cells secrete thyrocalcitonin which lowers the blood calcium, and named the cells “calcitonin cell (C-cell)”. C-cells are morphologically different from

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thyroid follicular cells: nonpolarized, pale-staining and larger than follicular cells. Besides, the most outstanding characteristics of the C-cells is their uneven and random distribution in the lateral thyroid lobes in most mammals except pigs and deer. The distribution is variable from species to species (Bläsher 1978). In man C-cells are present in the middle one third of the lateral lobes of thyroid glands (Wolfe et al. 1974). In the thyroid gland of normal adult gerbil, they occupy the posterior part of the lobes (Guilloteau et al. 1983) and in rats, they are localized chiefly in the center of the thyroid lobes both in an anterior-posterior and superior-inferior orientation (Stux et al. 1961; Peng et al. 1975). These localization of the C-cells in the thyroid lobes suggested that the C-cells might have different origin from the follicular cells. In fact, many investigators established that the C-cells have an embryonic origin rather than endoderm of primitive buccal cavity floor from which follicular cells are derived.

In 1927, Rogers first reported the fusion of ultimobranchial body with fetal primitive thyroid in rats. Godwin (1937) suggested that the C-cells might be derived from ultimobranchial body, and Pearse and Carvalheiro (1967) confirmed in their study that C-cells are derived from ultimobranchial body. Thereafter Polak et al. (1974), Kameda et al. (1976) declared that C-cells migrate from neural crest via ultimobranchial body.

Garel et al. (1981) and Jarzab et al. (1984) studied by immunofluorescence method when the cells were first detected in the rat fetal thyroid and the changes of absolute number of C-cells between prepartum and postpartum and the concentration of calcitonin in the thyroid extract. Above findings are insufficient for the understanding of the development and growth of C-cells. Moreover, little is known about the changes of the distribution and localization of C-cells in early developmental stages. Because the origin of C-cells is different from that of follicular cells and the mixing process of the two components of the thyroid gland is not meticulously understood, the aim of the present study is focused on the changes of C-cell pattern-distribution, localization, density-from prepartum to adult age.

MATERIALS and METHODS

1. Experimental animals

Normal healthy Sprague-Dawley rats were used regardless of their sexes. 3-, 7-, 15-day-old rats and adult rats weighing about 200 gm were anesthetized with ether. Each group consisted of five animals. The abdomen and thorax were opened and the heart was exposed. Primary fixation was done by perfusion of Bouin's fluid (Treilhou-Lahille et al. 1981) with prior saline perfusion in the case of adult rats and without prior saline perfusion in the case of 3-, 7-, 15-day-old rats. After dissection of cervical area, trachea with thyroid was exposed and excised. The esophagus was removed simultaneously for the orientation of the samples. The excised materials were secondly fixed with 4°C Bouin's fluid for 36 hours.

Fetal thyroides were also obtained. A female rat was caged with a male rat overnight. Next morning, the pregnancy was identified by the presence of vaginal plug and the day was counted as 0.5 day of gestation. Fetuses from 17.5-, 18.5-, 19.5-, 20.5- and 21.5-days of gestational age were gained from each pregnant rats and perfused with Bouin's fluid via umbilical veins. These fixed fetuses were dissected under the dissecting microscope and the thyroid with adjacent trachea, esophagus and vertebral column was exposed and excised. The specimens were secondly fixed in 4°C Bouin's fluid for 36 hours.

After fixation, the tissues were dehydrated, paraffin-embedded and horizontal serial sections were made at a thickness of 5 μm. 11 representative sections per each thyroid lobe were selected for immunohistochemical staining; one including superior pole, another including inferior pole, the others with same distance apart between superior and inferior poles.

2. Immunohistochemical staining

Tissue sections were stained by peroxidase-antiperoxidase method (Sternberger et al. 1970) which is known to be the most sensitive method to detect C-cells (Nunez and Gershon 1978).

The primary antiserum was rabbit anti-synthetic human calcitonin (DAKO, U.S.A) and is used at a dilution of 1:300 with 0.05 M PBS, pH 7.4. The incubation time was 18-24 hours at 4°C in the case of fetal tissues and 1 hour at room temperature with the others. The secondary antiserum was swine anti-rabbit Ig(DAKO) at a dilution of 1:100 with PBS. The incubation time was 30 minutes at room temperature. The tertiary antiserum was peroxidase-antiperoxidase complex (DAKO) at a dilution of 1:100 with PBS and the incubation time was 30 minutes at room temperature. 0.06% 3,3'-diamobenzidine solution with 0.005% H₂O₂.
which was freshly prepared was used as a substrate for coloring.

All sections were deparaffinized in xylene and hydrated in alcohol series. Antisera were applied in turn and tissues were washed after the application of each antiserum with 0.05M PBS, pH 7.4 (there times for 10 minutes each). Counterstaining was done with hematoxylin for 10-15 seconds and the stained sections were dehydrated, cleared and mounted as usual.

The specificity of immunohistochemical staining was tested by 1) replacing the primary antiserum with normal rabbit serum (DAKO) 2) omitting the second antiserum 3) comparison of the adjacent two sections (McMillan et al. 1974; Peng et al. 1975). The endogenous peroxidase activity was also tested by omitting all antisera from the process.

RESULTS

1. Specificity of staining

With the procedures for testing the specificity of this method, no staining was obtained. The specificity of this method was also supported by positive staining of the same cells in adjacent two sections. We obtained no positive staining after omitting all antigens, i.e., staining due to endogenous peroxidase activity was denied.

2. Appearance of C-cells

Immunoreactive C-cells to anti-calcitonin antibody were first observed in the 20.5-day-old fetuses, but in only one out of ten thyroid lobes. In the 21.5-day-old fetuses, all thyroid lobes revealed immunoreactive C-cells. The 20.5-day-old fetal thyroid also failed to show C-cells after prolongation of incubation time with the primary antiserum to 48 hours at 4°C or with condensation of primary antiserum to 1:20 dilution. Avidin–biotin–peroxidase complex method (Hsu et al. 1981; Childs and Unabia 1982) also failed to detect C-cells in the 20.5-day-old fetuses.

3. Distribution of C-cells

There was difference between the C-cell containing areas in the adult group and those in the remaining groups. In the adults, among 11 sections stained, all but uppermost 2–3 sections and lowermost 1–2 sections contained C-cells. In other words, C-cells were dispersed vertically through the middle 70%-80% of the entire thyroid length. In the 21.5-day-old fetuses and 3- and 7-day-old neonates, the vertical extents of C-cell containing area showed no difference with each other, but were more restricted than in adults (Fig. 1). In each thyroid lobe C-cells were most numerous in the sections of middle or slightly above middle of the lobe regardless of groups. Horizontally, C-cells were present restricted to the posteroentral area in the 21.5-day-old fetuses and 3- and 7-day-old neonates (Fig 2). In the adult group, however, they were detected throughout the thyroid parenchyma except for a small peripheral zone (Fig. 3). These observations suggested that the C-cell containing area was expanded both vertically and horizontally with aging. In the fetal and 3- and 7-day-old groups, the C-cell containing parenchymal area showed follicles smaller than those in the periphery and even not fully developed cell nests in the fetal group. Furthermore, loose connective tissue with blood vessels invaded from the mediastinal side of the thyroid lobe to separate the C-cell containing area and the medial portion into small pieces (Fig. 5). In the 15-day-old group, the C-cell containing area in horizontal direction extended more than in the fetal and 3- and 7-day-old groups, but not so much as in the adult group. No C-cell was observed in the isthmus regardless of age. Occasionally, there was a clear demarcation by connective tissue between areas with and without C-cells (Fig. 6).

4. Density of C-cells

In this study, we defined the unit area as 227 μm × 227 μm and counted C-cells in the unit area. The density of C-cells in each age group was 17.8±4.8, 16.2±4.9, 20.8±4.4, 22.6±6.1 and 54.7±9.3, respectively (Table 1). The difference between the fetal and 3-day-old group was of no
### Table 1. Developmental change of the C-cells in the rat thyroid gland

<table>
<thead>
<tr>
<th>Group</th>
<th>Cell density per unit area*</th>
<th>Intrafollicular C-cells</th>
<th>Singly appearing C-cells</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Total C-cells</td>
<td>Total C-cells</td>
</tr>
<tr>
<td>21.5-day-old fetus</td>
<td>17.8 ± 4.8</td>
<td>--</td>
<td>88.8%</td>
</tr>
<tr>
<td>3-day-old rat</td>
<td>16.2 ± 4.9</td>
<td>18.3%</td>
<td>84.1%</td>
</tr>
<tr>
<td>7-day-old rat</td>
<td>20.4 ± 4.4</td>
<td>20.0%</td>
<td>71.0%</td>
</tr>
<tr>
<td>15-day-old rat</td>
<td>22.6 ± 6.1</td>
<td>29.3%</td>
<td>54.7%</td>
</tr>
<tr>
<td>Adult rat</td>
<td>54.7 ± 9.3</td>
<td>30.3%</td>
<td>33.6%</td>
</tr>
</tbody>
</table>

* In this experiment, the unit area is defined as 227 \( \mu \text{m} \times 227 \mu \text{m} \).

statistical significance (p > 0.05). Therefore, the density of C-cell showed no significant change perinatally and began to increase gradually at 7 day postpartum.

### 5. Location of C-cells

C-cells were observed in intrafollicular or interfollicular locations. The proportions of intrafollicular C-cells to total number in the 3-, 7-, 15-day-old and adult groups was increasing with the value of 18.3%, 20.0%, 29.3% and 30.0%, respectively (Table 1). In the fetal group, the follicles were not fully developed, therefore, it was hardly possible to identify the locations of C-cells.

### 6. Grouping of C-cells

C-cells appeared both singly and in groups. The proportion of singly appearing C-cells to the total C-cells was decreased with aging (Table 1). In the fetal and 3-day-old groups, the majority of C-cells appeared singly while, in the adult group, they formed cell nests or cords. Cell nests and cords in the 3- and 7-day-old groups were principally composed of two C-cells. In the 15-day-old rats, cell groupings consisting of 3 C-cells were frequently observed. In the adult rats, cell nests and cords were usually composed of 3-5 C-cells and not infrequently up to 7-8 cells (Fig. 7). Intrafollicular C-cells usually appeared singly or forming cell cords but rarely cell nests.

### 7. Size and shape

In the fetuses, the size of C-cells was extremely variable from 5.6 \( \mu \text{m} \) to 11.3 \( \mu \text{m} \) in diameter. Small amount of cytoplasm formed a band around the nucleus (Fig. 8). In the adult group, C-cells were relatively uniform in size and 13.1 ± 2.3 \( \mu \text{m} \) in diameter. They were with abundant cytoplasm and larger than follicular cells. Though most C-cells were round or ovoid, the shape was highly variable: round, oval, triangular, polygonal, and spindle-shaped. Some C-cells possessed cytoplasmic process (Fig. 9).Others were crescent-shaped with their cytoplasm embracing another cell (Fig. 10).

### DISCUSSION

It is known that the amino acid sequence of rat thyrocalcitonin is similar to that of human calcitonin and that anti-human thyrocalcitonin antibody strongly crossreact with rat thyrocalcitonin in radiimmunoassay (Burford 1975). Moukhtar et al. (1974) reported the detection of C-cells in rat thyroids by the immunofluorescence method using anti-human thyrocalcitonin antibody. Thereafter, anti-human calcitonin antibody has been used to detect C-cells in rat thyroid glands (Martin-Lacave 1981; Treilhou-Lahille et al. 1981; Zabel 1982). In view of these facts, anti-human calcitonin antibody was also used in this study to detect C-cells in rat thyroids and the specificity of staining was ascertained.

In the human fetuses, the first immunoreactive C-cells were reported to be observed in the thyroid gland of a 14-week-old fetus (Leroyer-Alizon et al. 1980). In the rats, Alumets et al. (1980) observed that immunoreactive C-cells were rare in the fetal thyroid of Wistar rats and became gradually numerous after birth. Another report described that the immunoreactive C-cells were first detected in the 19.5-day-old rat fetus of Wistar strain(Garel et al. 1981). The different results of the above two experiments may be caused by the fixatives used in tissue preparation as was ascertained by Treilhou-Lahille et al. (1981); they observed that the picric acid, compared to the aldehyde fixative, destroyed the membrane of calcitonin-containing granules of C-cells in the mouse fetus to increase the stainability of C-cells by immunohistochemical method. In fact, Alumets et al. used formaldehyde and Garel et al. used Bouin’s fluid which contains...
the picric acid for tissue fixation. For this reason, Bouin's fluid was selected as a fixative in this study. However, the appearance of the first immunoreactive C-cells on the 21.5-day of gestation was still later than in the observation of Garel et al.. It is our opinion that such difference may be due to the strain difference of the experimental animals used. This was supported by Peng et al. (1976) who had pointed out the differences in calcitonin concentration of thyroid extracts from several different strains of rat.

The pattern of C-cell distribution in the thyroid lobe has been frequently described in several mammalian adults but rarely in fetuses or neonates. In the normal human neonate, C-cells are known to be restricted to a zone in the middle to upper one third of the thyroid lobe and deep into the parenchyme. The present result on the distribution pattern of C-cells in the fetal, 3- and 7-day-old groups are similiar to that of the human neonate in the aspect of their localization. According to Wolfe et al. (1974, 1975), the restricted pattern in human neonate was also observed in human adult. However, in this study, the restricted C-cell containing area in the neonate was expanding with aging up to almost entire thyroid parenchyme. Therefore, the significance of localization of C-cells in human neonate and rat neonate is thought to be different. Another difference between the two species of mammals is that the first appearance of immunoreactive C-cell in the human is far before birth, 14 weeks of gestation, whereas, in rats, just prior to birth, 21.5 days of gestation.

This study shows that C-cells in the adult rat are dispersed throughout the whole lateral lobe except in superior and inferior poles and extreme periphery of the lobe. Previous report by Stux et al. (1961) showed difference from ours in that C-cells were restricted to the center of the lobe. In their experiment, C-cells were detected by PAS-hematoxylin staining which was not as sensitive a method as immunohistochemical staining. Peng et al. (1975) also insisted that C-cells were in the central portion of the lobe. But, Bläsher(1978) obtained the same result as ours which showed that C-cells were distributed throughout the thyroid gland except for a small peripheral zone and the polar regions.

The area where C-cells are first detected is the posteroentral part of thyroid parenchyme, where the remnant of the ultimobranchial body persists after fusion with primitive thyroid(Rogers 1927). This finding may support and coincide with the theory that C-cells are derived from ultimobranchial body (Godwin 1937; Pearse and Carvalheira 1967). Little is known about the mechanism how the C-cells diffuse out with aging in rats.

The density of C-cells shows increasing tendency with aging in this study. The presented numbers are of course not absolute numbers. But they will be sufficient to reflect the changing tendency with aging. Considering the increase of density with that of C-cell containing area, we can speculate that the rate of increase in absolute number of C-cells exceed that in density increase.

Our results showed that the majority of C-cells appear singly in the fetal and 3-day-old groups while the ratio of C-cells forming cell nests or cords is increasing with aging. The same result was previously reported by Wechbanjong et al. (1979). Wechbanjong et al. observed that C-cells in the mouse thyroid were present both singly and in groups, except in the 1-day-old mice in which singly appeared C-cells were predominant. Many other investigators also reported the grouping of C-cells in adult rats (Bläsher 1978; Peng et al. 1975; Stux et al. 1961) and predominence of singly appearing C-cells in the neonatal rats(Christov et al. 1972). These reports coincide with ours.

The relation of C-cells with follicles has been a matter of debate. C-cells were reported to be always inside the basement membrane of follicles in electron microscopic examination of the rat thyroid gland(Young and Leblond 1963). Sarkar and Iser(1963) reported the same result by PAS-hematoxylin staining. But above results are somewhat doubtful for their methods were unsuitable. Wolfe et al. (1974, 1975) reported different result in their experiment using immunohistochemical method. They observed that C-cells were both intrafollicular and interfollicular in the human adult, the latter being about 75%. This result is very similar to ours. We determined that C-cells were both intrafollicular and interfollicular, the latter being 69.7%–81.7%. In mice, C-cells are also reported to be present both intrafollicular and interfollicular regardless of age (Wechbanjong et al. 1979; Treihou-Lahille et al. 1981).

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면역조직화학적 방법을 이용한 흰쥐 감상선에서의 C-cell의 발생 및 성장에 관한 연구

향일일, 이병관, 차중익, 장가용, 이광호, 성기준

Sprague-Dawley체 흰쥐의 개체발생 및 성장에 따른 감상선 C-cell의 변화를 알아보기 위하여 임신 17.5일, 18.5일, 19.5일, 20.5일, 21.5일의 흰쥐와 생후 3일, 7일, 15일 및 체중 200g 내외의 성숙 흰쥐 대상으로 면역조직화학적 염색을 시행하였다. 염색한 조직결리는 감상선조직을 포함하는 수평 연속절편 중 균등한 간격의 조직절편 11장을 선택하였으며 면역조직화학적 염색 후 hematoxylin으로 바조염색하여 광학현미경에서 관찰하여 다음과 같은 결과를 얻었다.

1. 면역조직화학법으로 염색된 C-cell은 임신 20.5일 군에서 처음으로であり게 관찰되었으나 하나의 감상선엽에서만 관찰되었고 임신 21.5일 군에서는 모든 감상선엽에서 관찰되기 시작하였다.

2. 감상선엽 내에서 C-cell이 분포한 영역은 임신 21.5일 군에서는 위엽의 후중앙부였으나 연령증가에 따라 점차 확산되어 성숙군에서는 양엽을 포함한 일부와 주변부를 제외한 모든 감상선엽에서 C-cell이 관찰되었다.

3. C-cell의 밀도는 임신 21.5일 태어, 생후 3일, 7일, 15일 및 성숙군에서 각각 17.8±4.4개, 16.2±4.9개, 20.8±4.4개, 22.6±6.1개, 54.7±9.3개로 관찰되어 연령증가에 따른 C-cell밀도의 증가를 보였다.

4. 전체 C-cell 중 단독으로 존재하는 C-cell의 비율은 연령증가에 따라 급격히 감소하였으며 임신 21.5일 태어, 생후 3일, 7일, 15일 및 성숙 군에서 각각 88.8%, 84.1%, 71.9%, 54.7%, 33.6%로 나타났다.

5. 성숙군에서 C-cell은 그 형태가 매우분 원형, 타원형이었으나 소수는 삼각형, 타원형 및 납추형으로 매우 다양하였으며 크기는 13.1±2.3μm이었다. 임신 21.5일 태어군의 C-cell은 크기가 5.6μm±11.3μm로 다양하였으며 세포질의 양이 성숙군에 비하여 적었다.
EXPLANATIONS FOR FIGURES

Fig. 2. Transverse section of the midportion of the 3-day-old rat thyroid. Immunoreactive C-cells are well localized in the posterocentral portion of the section. x100.

Fig. 3. Transverse section of the midportion of adult rat thyroid. Immunoreactive C-cells are spread throughout the entire section. x100.

Fig. 4. Transverse section of midportion of the 21.5-day-old fetus thyroid. Groups of follicular cells are dispersed in the loose connective tissue (arrow head). Follicles are not completely formed (PAS-hematoxylin staining). x400.

Fig. 5. Transverse section of the 3-day-old rat thyroid. Thyroid parenchyma is separated into small portions by vascular connective tissue. A large vessel is seen medial to the thyroid. x200.

Fig. 6. Thyroid section from adult rat. There is a clear demarcation between the areas with and without C-cells. x200.

Fig. 7. Thyroid section from adult rat. Numerous C-cells are observed in one cell nest (arrow head). x1,000.

Fig. 8. Thyroid section from the 21.5-day-old fetus. The cytoplasm of the stained C-cell appears as a band-like rim around its nucleus. x1,000.

Fig. 9. Thyroid section from adult rat. Spindle-like cytoplasmic process of a C-cell is seen (arrow head). x1,000.

Fig. 10. Thyroid section from adult rat. A crescent-shaped C-cell is shown (arrow head). It seems to embrace another cell. x1,000.