

## Role of Adenohypophyseal Mixed Cell-follicles in Age Estimation

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In this study we used paraffin-embedded human pituitary obtained from 248 autopsy cases and identified mixed cell follicles by the immunohistochemical method. We examined the number and size of the mixed cell follicles, and the ratio of each component cell of these follicles, in the anterior pituitary at various age groups. The number of follicles increased with age, and the size of the follicles also tended to enlarge with age. Statistical analysis showed that a high correlation existed between age and the number or the size of the mixed cell-follicles formed by various adenohypophyseal cells. In addition, when the proportions of the different cell types that formed the follicles were examined, sex differences were observed with aging for the GH cells, the PRL cells, and the gonadotroph (GTH) cells, while no changes were observed with aging in both men and women for the ACTH cells and TSH cells. These results indicate that the number, size, and ratio of each component cell of follicles in the anterior pituitary are adequately applicable for the purpose of age estimation in routine forensic medicine.

**Key words:** mixed cell-follicle, human anterior pituitary, age estimation

The age estimation of an unidentified dead body is an important task in forensic medicine. Various methods of age estimation based on morphological studies have been attempted in the past, including measurement of the height of the apex of the medullary cavity of the humerus [1, 2], the state of fusion of the cranial suture [3], observation of the eruption stage of deciduous teeth and permanent teeth [3], the degree of tooth attritions [4], and the presence of white hair in the scalp and pubic hair [5]. However, these methods give an estimated age, and the estimated age does not correspond to the actual age in many instances.

Recently, anatomical and physiological studies have

reported a high detection rate of follicles in the adenohypophysis of aged vertebrates [6, 7]. In particular, Ogawa *et al.* [7] observed a large number of follicles in porcine adenohypophysis and reported the physiological significance of this phenomenon. However, as far as we know, there have been no reports regarding changes in the adenohypophyseal follicles with increasing age.

In the present study, we examined the number and size of mixed cell-follicles, and the ratio of each component cell of these follicles in the human anterior pituitary at various age groups. We also analyzed whether these changes correlated with age, and whether the obtained results could be used in age estimation.

### Materials and Methods

Morphologically intact adenohypophyses were

obtained from 248 forensic and systemic autopsied cases (195 men and 53 women, aged 12 to centenarian) without any endocrinological or metabolic diseases, alcoholic diseases, or malignant tumors. After the pituitary was harvested, the dura was removed under a stereomicroscope. The specimen was fixed by shaking in 10 % phosphate-buffered formalin for 3 days at 4°C. The fixed tissue was then embedded in paraffin. The paraffin-embedded tissue was divided along the frontal plane into the anterior 1/3 portion, middle portion, and posterior 1/3 portion. Four- $\mu\text{m}$  serial sections were prepared from various regions of the hypophysis for enumerating the number and size of follicles, and 200- $\mu\text{m}$  sections were prepared to determine the real diameter of the follicles. After deparaffinization with xylene, the sections were processed through a series of alcohol and down to water. Since the follicle contents are known to be PAS (periodic acid Schiff)-positive [7], the first slide of the serial sections was stained with PAS. The subsequent sections were used in immunohistochemical studies to identify the cells that form the follicles. The following primary antibodies were used: rabbit anti-human growth hormone (GH) (22 K) serum (diluted 1:8,000; supplied by NIDDK, MD, USA), rabbit anti-adrenocorticotrophic hormone (ACTH) (1-24 N-terminal) serum (diluted 1:8,000; supplied by the Department of Anatomy, Jikei University School of Medicine, Tokyo, Japan) [8], rabbit anti-human follicle-stimulating hormone (FSH) serum (diluted 1:7,000; Scantibodies Laboratory, Inc., CA, USA), rabbit anti-human prolactin (PRL) serum (diluted 1:8,000; Biogenesis, New Hampshire, UK), and rabbit anti-human thyroid-stimulating hormone (TSH) serum (diluted 1:5,000, supplied by NIDDK, MD, USA). Immunostaining was conducted by the avidin biotin complex (ABC) method, and color was developed with 3,3'-diaminobenzidine (DAB). The next serial section was used in a double-staining study using anti-human ACTH antibodies and anti-human S-100 protein antibodies (Polyclonal, Whole (both  $\alpha$  and  $\beta$  subunits), 1:5,000 diluted, supplied by the Department of Anatomy, Jikei University, School of Medicine) [9]. Color was developed with naphthol (4-Chloro-1-naphthol) and DAB. When necessary, hematoxylin and eosin (HE) staining was performed to observe the morphology of the follicles and the follicle-forming cells.

#### *Identification of mixed cell-follicle[s].*

The pars intermedia follicles excluded from measurement showed the following morphology: surrounding the folli-

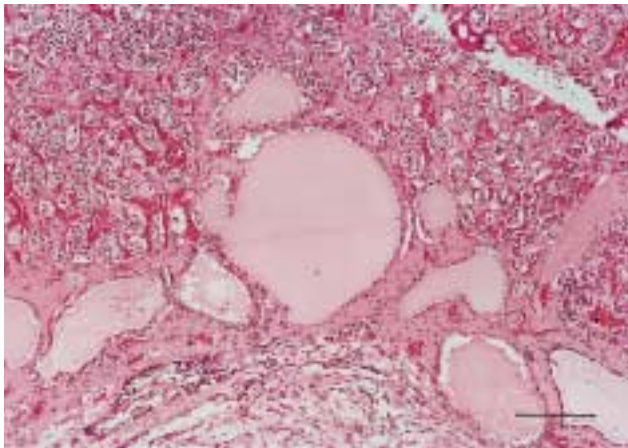
cle, flattened cells juxtaposed and joined together in a ring form. At some sites, the follicle-forming cells encompassing the follicle formed a multi-cell layer, and a portion of the cytoplasm of the flattened cell extended in between the follicle-forming cells. In comparison, the mixed cell-follicles measured in the present study were formed mainly by 1 to 3 layers of eosinophilic cells that encircled the follicles. Occasionally, some of the follicle-forming cells showed a process-like structure that extended in between the follicle-composing cells.

For the pars intermedia follicles, the flattened cells that joined together in a ring form around the follicle by HE staining were found to contain S-100 protein or ACTH by immunohistochemical study, and S-100-positive cells were found interposed between the ACTH-positive cells.

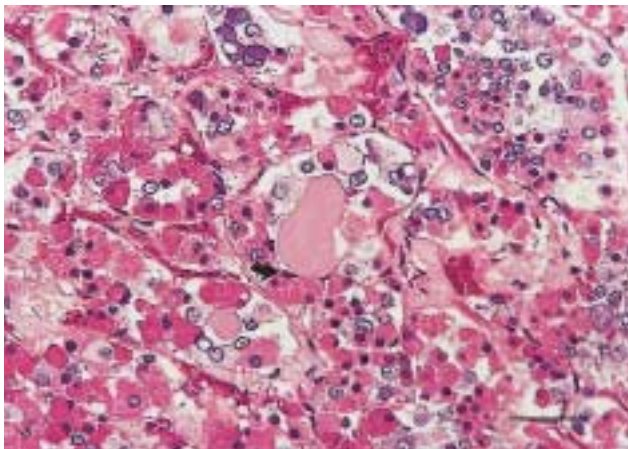
For the cells forming mixed cell-follicles, however, the eosinophilic cells were mainly GH-secreting cells, and the remaining cells were positive for various adenohypophyseal hormones and the S-100 protein.

***Number of mixed cell-follicles.*** The total number of follicles (pars distalis follicles + pars intermedia follicles) was determined using the PAS-stained sections by a color image analyzer, CIA-102 (Olympus, Tokyo, Japan). Since the present study aimed to measure only the mixed cell-follicles present in the adenohypophysis, follicles of the pars intermedia formed exclusively only by ACTH-positive and S-100-positive cells [10] were excluded from measurement. In the actual measurements, follicles less than 5  $\mu\text{m}$  were below the discrimination limit and were not measured. Furthermore, since the sizes of the follicles varied depending on age, the cell number correction formula reported by Yoshimura and Ishikawa [11] was used to convert the enumerated number measured to a more accurate number. In the enumeration of the number of follicles in the present study, the larger the size of the follicles, the greater the over-estimation. Therefore, to find the real number of follicles from one section, we used the correction equation of Yoshimura and Ishikawa [11], which corrects for overlapped counting of adjacent and near sections. The numbers of follicles detected in the hypophysis per male or female subject were stratified by age group and analyzed by the regression method.

***Size of mixed cell-follicles.*** We also analyzed the age-related change in the size of follicles per subject. Follicles formed by cells positive for any of the above hormones were subjected to size determination



a



b

**Fig. 1** (a), Hematoxylin-eosin stained section of pars intermedia follicles (Bars indicate  $200\ \mu\text{m}$ ). Surrounding the follicle, flattened cells juxtapose and join together in a ring form. Calcification was sometimes observed inside the follicles. (b), Hematoxylin-eosin stained section of mixed cell-follicles. When the mixed cell-follicle consists of mainly eosinophilic cells and one to several layers of eosinophilic, basophilic, and chromophobic cells encompassing the follicle, cells that penetrate between the follicle-forming cells may be observed (arrow). Bars indicate  $100\ \mu\text{m}$ .

using a color image analyzer, CIA-102 (Olympus, Tokyo, Japan). The real diameters of the follicles were analyzed using a confocal laser microscope (Zeiss, LSM-510, Jena, Germany). For analysis, a 40-x objective lens was used, and the follicles were traced at a discrimination limit of  $5\ \mu\text{m}$ . If the PAS-positive portion was not in contact with the inner wall of the follicle, the distance up to the inner wall of the follicle-forming cell was measured to determine the follicle size. The correlation

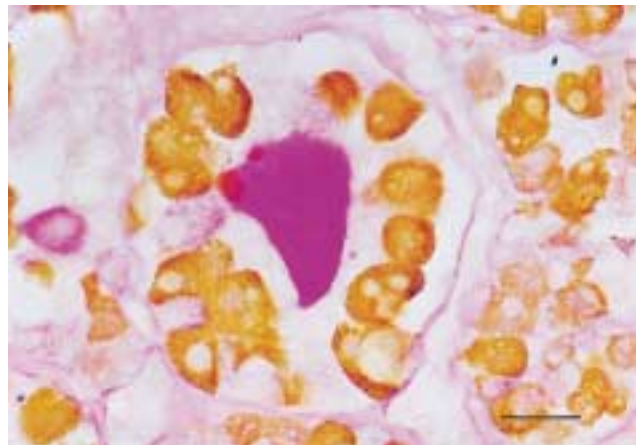
between age and the size of the follicle was analyzed separately for men and women.

**The changes of component cells in mixed cell-follicles.** We investigated whether there were changes in proportion of the various cell types forming the mixed-cell follicles with aging, using serial sections immunostained with various antibodies.

## Results

**Structure of follicle and follicle-forming cells.** The pars intermedia follicles are shown in Fig. 1a. Surrounding the large follicles are flattened cells juxtaposed and joined together in ring form. In comparison, the mixed cell follicles were formed of 1 to 3 layers of eosinophilic, basophilic, and chromophobic cells that encircled the follicles (Fig. 1b).

For cells forming the mixed cell-follicles, in men, the eosinophilic cells were mainly GH-secreting cells (Fig. 2 and Fig. 3a), however in women, the GH cells appeared to be mainly the cell type (55%) in younger subjects, and these cells decreased to approximately 37% in the elderly (Fig. 3b). In men, prolactin cells occupied approximately 18% in young subjects (aged 20 to 30 years), and decreased gradually to approximately 6% in the elderly (Fig. 3a). However in women, the proportion was 25% in the younger subjects, and decreased gradually to approximately 12% in the elderly (Fig. 3b). In men,



**Fig. 2** Double-staining with PAS and immunostaining using anti-human growth hormone (GH) antibodies stained of mixed cell-follicles. The follicle that stained reddish-purple by PAS is surrounded by cells which mainly secrete GH. Bars indicate  $30\ \mu\text{m}$ .

gonadotrophs occupied approximately 3% in young subjects, and increased gradually to 23% in the elderly (Fig. 3a). In women, however, the proportion remained almost unchanged at approximately 7% from the 20s to the 40s, but increased abruptly to 18% in the 50s and 40% in the elderly (Fig. 3b). However, the ACTH cells and TSH cells did not change with aging in both men and women (Figs. 3a and 3b).

In the young age groups of teens and twenties, mixed cell-follicles were localized in the adenohypophyseal sites near the pars intermedia and at the border on the ventrolateral side and dural side of the adenohypophysis (Fig. 4a). In comparison, the follicles were found diffusely throughout the adenohypophysis in elderly subjects in their seventies and eighties (Fig. 4b). In the mixed cell-follicles, although no calcification was observed

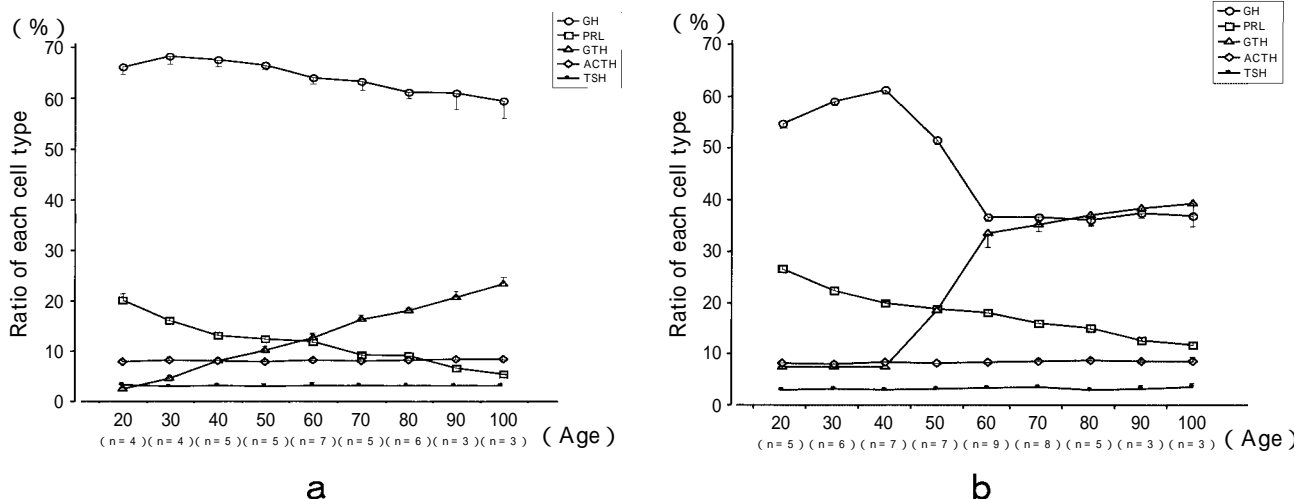


Fig. 3 Graph showing the relationship between age and changes in the ratio of each cell type forming mixed cell-follicles in men (a) and women (b).

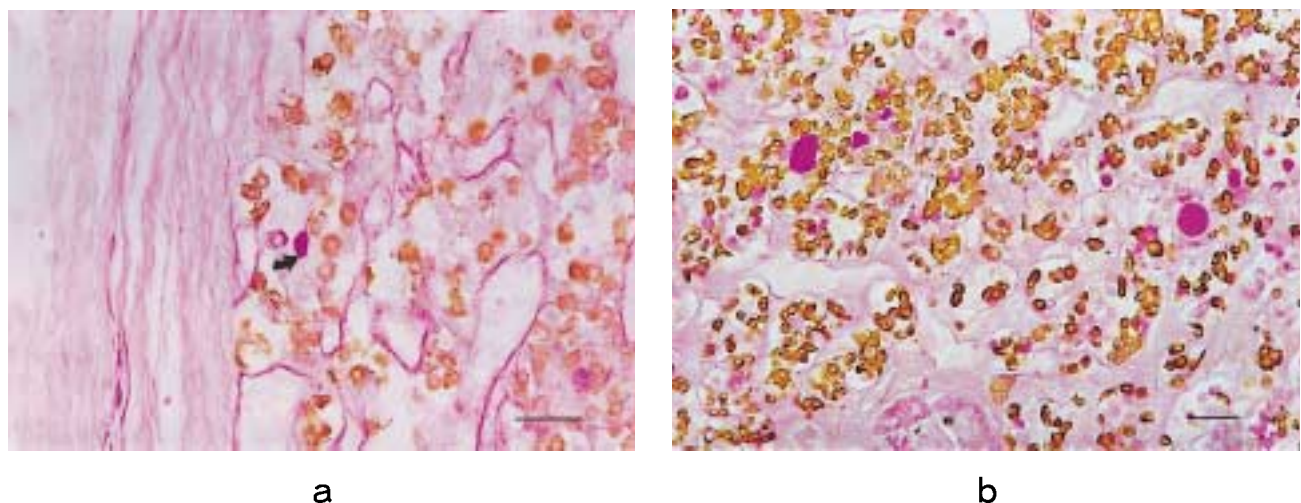
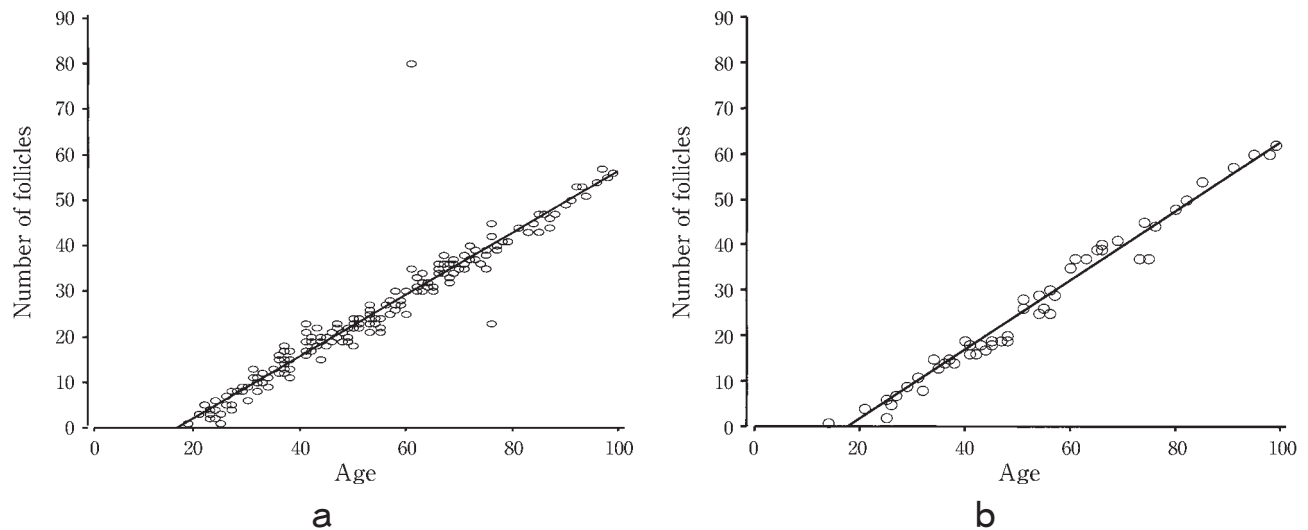


Fig. 4 Doubled-stained with anti-GH antibodies and PAS. (a), Mixed cell-follicle in the adenohypophysis of a man in his twenties. In young subjects, a few round or ovoid follicles appeared on the dural side or the border with the pars intermedia. The capsule is observed on the left side. Arrow shows follicular lumen (colloid) (Bars indicate 50 μm). (b), Mixed cell-follicles in the adenohypophysis of a man in his seventies. The size of the follicles is larger than those in young subjects. Bars indicate 80 μm.

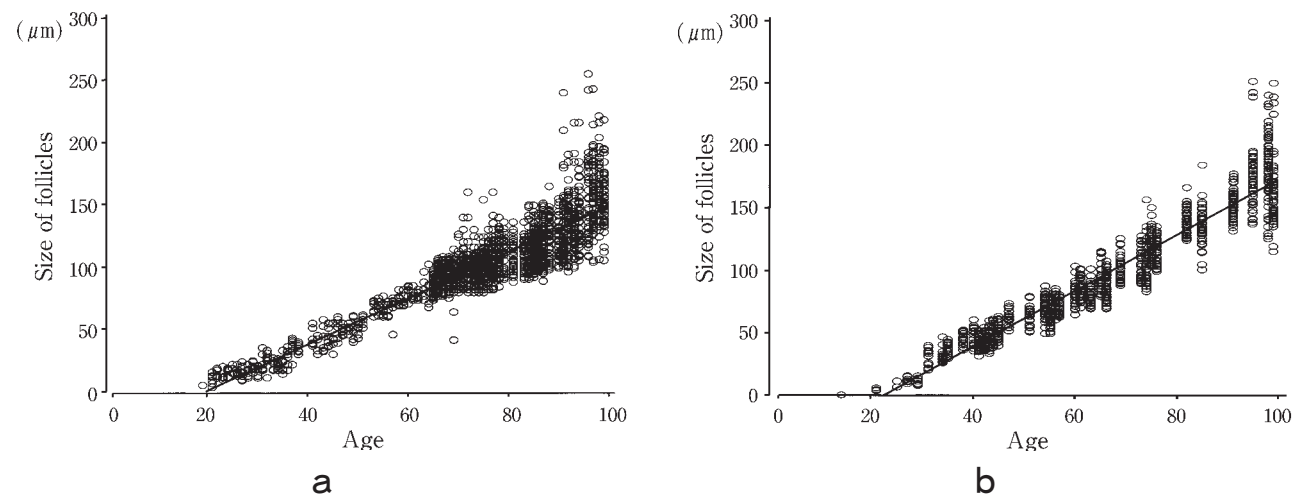
inside the follicle, sometimes the presence of small vacuoles in the peripheral area of the colloid were identified.

**Relationship between age and number of follicles.** When the relationship between age and the number of mixed-cell follicles per subject was depicted graphically (Fig. 5), follicles larger than the discrimination limit of  $5\ \mu\text{m}$  were not found in the adenohypophysis

in males from 0 to 18 years of age, and in females from 0 to 13 years of age. However, the number of mixed cell-follicles appeared from the late teens, and the number increased with age for both men and women. In men, the number of mixed cell-follicles increased to approximately 40 in the seventies, and to almost 60 in the nineties. In both men and women, a positive correlation was observed between age and the number of follicles. The regression



**Fig. 5** Graph showing the relationship between age and the changes in the number of mixed cell-follicles (per subject) found in the adenohypophysis in men (a) and women (b). Both showed a tendency for increase in the number of follicles accompanying aging.



**Fig. 6** Graph showing the relationship between age and the changes in the size of all mixed cell-follicles (per subject) found in the adenohypophysis per subject in men (a) and women (b). Both showed a tendency for linear increase in the size of the follicles accompanying aging.

equations were  $y = 0.678x - 11.343$  (x: age (year), y: number of follicles;  $r = 0.950$ ) in men (Fig. 5a), and  $y = 0.759x - 13.42$  (x: age (year), y: number of follicles;  $r = 0.989$ ) in women (Fig. 5b), both showing a tendency for a linear increase in the number of follicles accompanying an age increase.

**Relationship between age and size of follicles.** The relationship between age and the size of the mixed-cell follicles in the adenohypophysis was analyzed graphically (Fig. 6). In men, the size was around  $20 \mu\text{m}$  in the twenties, and increased to over  $200 \mu\text{m}$  in the nineties. A similar trend of increasing follicular size with age was also observed in women. The mixed cell-follicles observed in elderly women tended to be slightly larger than those in elderly men, although this aspect was not analyzed statistically in the present study. In the eighties, while the sizes were around  $110 \mu\text{m}$  in men, they were around  $130 \mu\text{m}$  in women. For both men and women, a positive correlation was observed between age and the size of the follicles. The regression equations were  $y = 1.856x - 36.606$  (x: age (year), y: size of follicles ( $\mu\text{m}$ );  $r = 0.879$ ) in men (Fig. 6a), and  $y = 2.231x - 49.927$  (x: age (year), y: size of follicles ( $\mu\text{m}$ );  $r = 0.956$ ) in women (Fig. 6b), both showing the tendency for linear increase in the size of the follicles accompanying age increase.

## Discussion

In general, the hypophysis in humans has been reported to change in external appearance to a boat shape in the aged [12]. However, as far as we know, there have been no reports stating that the histological changes in the hypophysis may be used to estimate age.

The detection of PAS-positive colloid has been reported in the pars distalis, pars intermedia, and pars tuberalis of the hypophysis in aged vertebrates belonging to various species [6, 7]. The follicles present in the hypophysis can be divided into 3 types depending on the differences in the follicle-forming cells. The first type is the follicles analyzed in the present study, which are present in the adenohypophysis and are formed by cells secreting adenohypophyseal hormones such as GH and TSH, or containing S-100 protein. Gon *et al.* [13] named this type of follicles in rats mixed cell-follicles, and reported that 50% of the follicle-forming cells are GH secreting cells. From the results of the present study, GH cells were also apparently more abundant than the other adenohypophyseal cells in humans. The number of follicles that

include GH cells increases with aging, but the ratio of GH cells in the follicles decreases. The reason for the decrease in the proportion of GH cells in the mixed cell follicles remains unknown.

The second type is generally called follicle stellate-cells. They are found in the adenohypophysis, and the follicle-forming cells are composed of S-100 protein-positive cells only. There are no other cells producing adenohypophyseal hormones [14]. The third type is the group of follicles called pars intermedia follicles [10], which are localized mainly in the pars intermedia of the adenohypophysis, and are formed exclusively from cells that produce ACTH and contain the S-100 protein.

In the present study, follicles of the follicle-stellate cell type (the second type) were not included in the measurements from the beginning because they are irregularly shaped follicles generally smaller than  $5 \mu\text{m}$  (the diameter of the colloid), and can only be identified under an electron microscope. In addition, pars intermedia follicles were not measured because of large variations in the number and size when used for the purpose of age estimation (data not shown).

The follicles that were measured in this study varied in size from 5 to  $200 \mu\text{m}$ . Follicles smaller than  $5 \mu\text{m}$  were considered below the discrimination limit and were not measured, because they cannot be distinguished from the cut end of basophils such as TSH, ACTH, and GTH cells which are PAS-positive. In addition, we conducted a correction of the enumerated number. In  $4\text{-}\mu\text{m}$  sections, a  $10\text{-}\mu\text{m}$  follicle appears in 3 to 4 sections, while a  $200\text{-}\mu\text{m}$  follicle is contained in 50 to 51 sections. Furthermore, a tendency of change in follicle size with age was observed. Considering these factors, the possibility of repeatedly measuring a follicle exists, and therefore a method for more accurate estimation of the number of follicles is required. We used the cell (structures) number correction formula previously reported by Yoshimura and Ishikawa [11], and converted the enumerated number into a more accurate number of follicles. This correction equation is excellent in that it can be used regardless of the shape of the structures (or cells) observed in a section sample. When the structures or cells exist in various sizes, over-estimation is greater with an increase in size. Therefore, the use of this equation is necessary to determine the real number in the specimen from the numbers counted in a section. Under these conditions, we found that the number of follicles increased with age in both men and women.

In estimating the size of the follicles, we used a confocal laser microscope to observe sections 200  $\mu\text{m}$  in thickness, and measured the greatest diameter of each follicle. The results also showed a tendency of expansion of follicular size with age, similar to the results for the number of follicles.

Recently, Ogawa *et al.* [15] analyzed the follicular colloid in the hypophyses of senescent porcine. They isolated 4 proteins ranging in molecular size from 26 to 60 kD, and identified the main component as clusterin, a type of sulfated glycoprotein [16-18]. However, there are no reports that precisely identify the follicular contents in humans, and the question of whether the follicle-forming cells affect the follicular content remains unanswered. In the present study, we found distinct sex differences accompanying age increase in the proportions of various adenohypophyseal hormone-secreting cells forming the follicles. The GH cells decreased with aging in both men and women, but the percent decrease was greater in women than in men. Similarly, prolactin cells also decreased in both men and women, but the percent decrease was greater in men than in women. Gonadotrophs cells increased with aging in both men and women, but while men showed a gradual increase, women stayed unchanged until the 40s, and then showed an abrupt increase. However, the ACTH cells and TSH cells showed no sex difference and no change in proportion accompanying aging. The biphasic changes of gonadotrophs cells in women are considered to be caused by menopause. However, the absence of such change patterns in men is an interesting finding. The reason why the change in GH cells is greater in women than in men also remains unknown.

The present study showed a clear correlation between age and the number of follicles, and also between age and the size of the follicles, although further studies are required to elucidate the reason for the linear increase and expansion of the mixed cell-follicles in the adenohypophysis. The number and size of the mixed cell-follicles determined by the PAS reaction are very useful for age estimation, because the PAS reaction is available to the pituitary regardless of the time that has elapsed after death.

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