

ELECTRON MICROSCOPIC RADIOAUTOGRAPHIC STUDY ON MITOCHONDRIAL RNA SYNTHESIS IN ADRENOCORTICAL CELLS OF AGING MICE

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Abstract: *In order to study the aging changes of intramitochondrial RNA synthesis of mouse adrenocortical cells, 10 groups of developing and aging mice, each consisting of 3 individuals, total 30, from fetal day 19 to postnatal newborn at day 1, 3, 9, 14, adult at month 1, 2, 6 and senescent animals at month 12 (year 1) and 24 (year 2) were injected with ³H-uridine, an RNA precursor, sacrificed 1 hr later and the adrenal tissues were fixed and processed for electron microscopic radioautography. On electron microscopic radioautograms obtained from each animal, the number of mitochondria per cell, the number of labeled mitochondria with ³H-uridine showing RNA synthesis per cell and the mitochondrial labeling index in each adrenocortical cells, in 3 zones, were counted and the results in respective aging groups were compared with each others. From the results, it was demonstrated that the number of mitochondria per cell in 3 zones, the zona glomerulosa, fasciculata and reticularis of respective mice at various ages increased from fetal day 19 to postnatal month 1 reaching the plateau from month 1 to 24 due to development and aging of animals, respectively, while the number of labeled mitochondria per cell with intramitochondrial RNA synthesis incorporating ³H-uridine increased from fetal day 19 to postnatal month 2, reaching the maxima and decreased slightly from month 6 to month 24. The mitochondrial labeling index calculated from the numbers of mitochondria per cell and the numbers of labeled mitochondria increased from fetal day 19 to postnatal day 3, 9 and 14, reaching the maximum and decreased gradually from month 1 and again increased at month 2 and decreased to month 24. It was shown that the activity of intramitochondrial RNA synthesis in the adrenocortical cells in developing and aging mice changed due to aging of individual animals.*

Key words: Mitochondria, Mouse adrenal cortex, EM radioautography, RNA synthesis

INTRODUCTION

Intramitochondrial nucleic acid syntheses, both DNA and RNA, in mammalian and avian cells were first demonstrated morphologically by the present author by means of electron microscopic radioautography in primary cultured cells of the livers and kidneys of mice and chickens in vitro [1] and then in some other established cell lines such as HeLa cells [2, 3] or mitochondrial fractions prepared from in vivo cells [4]. It was later commonly found in various cells and tissues not only in vitro obtained from various organs

in vivo [5, 6, 7, 8], but also in vivo cells of various organs such as the salivary glands [9], the liver [10-22], the pancreas [23, 24], the trachea [25], the lung [26], the kidney [27], the testis [28, 29], the uterus [30, 31], the adrenal [32-34], the brain [35], and the retina [36-40] of mice, rats and chickens. The relationship between the intramitochondrial DNA synthesis and cell cycle was formerly studied and it was clarified that the intramitochondrial DNA synthesis was performed without nuclear involvement [2]. However, the relationship between the aging of individual animals and the DNA synthesis in

respective cell types in these organs has not yet been clear. Recently, the relationship of both the DNA synthesis and RNA synthesis to the aging of animals was first clarified in the hepatocytes of mice [12-14]. Later, the relationship of the DNA synthesis to the aging of animals in the adrenocortical cells was also clarified [32,33]. However, the relationship of the RNA synthesis to the aging of animals in the adrenocortical cells has not yet fully been clarified [34]. This paper deals with the relationship between the RNA synthesis and the aging in the adrenocortical cells of mice *in vivo* at various developmental stages from fetal day 19 to postnatal month 2 and further to adult and senescent stages up to month 24 (year 2) during aging by means of electron microscopic radioautography as a part of serial studies on special cytochemistry [41] and radioautography [42].

MATERIALS AND METHODS

The experimental animals: The adrenal tissues were obtained from 10 groups of developing and aging normal ddY strain mice, from fetal day 19 to postnatal newborn at day 1, 3, 9, 14, adult at month 1, 2, 6, 12 and 24, each consisting of 3 litter mates of both sexes, total 30. The embryonic age was based on observation of the vaginal plug of the female mice (vaginal plug=day 0). All the animals were housed under conventional conditions and bred with normal diet (mouse chow Clea EC2, Clea Co., Tokyo, Japan) with access to water *ad libitum* in our laboratory. They were administered with ³H-uridine, RNA precursor, and the adrenal tissues were fixed and processed for electron microscopic radioautography. All the procedures used in this study concerning the animal experiments were in accordance with the guidelines of the animal research committee of Shinshu University School of Medicine, Matsumoto, Japan, where this experiment was carried out, as well as the principles of laboratory animal care in NIH publication No. 86-23 (revised 1985).

Electron microscopic radioautography: All the animals were injected intraperitoneally with ³H-uridine (Amersham, England, specific activity 877 GBq/mM) in saline, at 9 a.m., one hour before sacrifices. The dosage of injections was 370 KBq/gm body weight. The animals were perfused at 10 a.m., one hour after the injection, via the left ventricles of the hearts with 0.1 M cacodylate-buffered 2.5% glutaraldehyde under Nembutal (Abbott Laboratories, Chicago, ILL, USA) anesthesia. The right adrenal glands were taken out, excised and 3 small pieces of

the adrenal tissues (1 mm x 1 mm x 1 mm) were immersed in the same fixative at 4°C for 1 hr., followed by postfixation in 1% osmium tetroxide in the same buffer at 4°C for 1 hr., dehydrated in graded series of ethanol and acetone, and embedded in epoxy resin Epok 812 (Oken, Tokyo, Japan).

For electron microscopic radioautography, semithin sections at 0.2µm thickness, thicker than conventional ultrathin sections containing more radiolabeled compound than ultrathin sections in order to shorten the exposure time, were cut in sequence on a Porter-Blum MT-2B ultramicrotome (Dupont-Sorvall, Newtown, MA, USA) using glass knives. The sections were collected on collodion coated copper grid meshes (VECO, Eerbeek, Netherlands), coated with Konica NR-H2 radioautographic emulsion (Konica, Tokyo, Japan) by a wire-loop method [23, 41-44]. They were stored in dark boxes containing silica gel (desiccant) at 4°C for exposure. After the exposure for 10 months, the specimens were processed for development in freshly prepared gold latensification solution for 30 sec at 16°C and then in fresh phenidon developer for 1 min at 16°C in a water bath, rinsed in distilled water and dried in an oven at 37°C overnight, stained with lead citrate solution for 3 min, coated with carbon for electron microscopy. The electron microscopic (EM) radioautograms were examined in a JEOL JEM-4000EX high voltage electron microscope (JEOL, Tokyo, Japan) at an accelerating voltage of 400kV for observing thick specimens [42].

Quantitative analysis of electron micrographs: For quantitative analysis of electron micrographs, twenty EM radioautograms showing cross sections of adrenocortical cells sectioned through the centers of their nuclei and cell bodies selected at random from each group, based on the electron microscopic photographs taken after observation on at least 100 adrenocortical cells from respective animals, and at least 10 cells from respective zones, i. e. zona glomerulosa, zona fasciculata and zona reticularis, were analyzed to calculate the total number of mitochondria in each adrenocortical cell in respective zones, and the number of labeled mitochondria covered with silver grains by visual grain counting.

On the other hand, the number of silver grains in the same area size as a mitochondrion outside cells was also calculated in respective specimens as background fog, which resulted in less than 1 silver grain (0.03/mitochondrial area) almost zero.

Therefore, the grain count in each specimen was not corrected with the background fog. From all the data thus obtained the averages and standard deviations in respective aging groups were computed with a personal computer. The data were statistically analyzed using variance and Student's t-test. The differences were considered to be significant at P value < 0.01 .

RESULTS

Morphological observations: The adrenocortical tissues obtained from ddY strain mice at various ages from embryonic day 19 to postnatal day 30, consisted of 3 layers, zona glomerulosa, zona fasciculata and zona reticularis, showed gradual development. At embryonic day 19 and postnatal day 1, the adrenocortical cells were composed mainly of polygonal cells, while the specific orientation of the 3 layers, zona glomerulosa, zona fasciculata and zona reticularis, was not yet well established. At postnatal day 3, orientation of 3 layers, zona glomerulosa, zona fasciculata and zona reticularis, became evident. At postnatal day 9 and 14, the specific structure of 3 layers was completely formed and the arrangements of the cells in respective layer became typical especially at day 14. Observing the ultrastructure of the adrenocortical cells, cell organelles including mitochondria were not so well developed at perinatal and early postnatal stages from embryonic day 19 to postnatal day 3. However, these cell organelles, mitochondria, endoplasmic reticulum, Golgi apparatus, appeared well developed from the juvenile stage at postnatal day 14 to the adult stages at postnatal month 1, month 2, month 6 (Figs. 1-3) and further to the senescent stage at month 12 (Figs. 4-6) and 24.

The zona glomerulosa (Figs. 1, 4) of mouse adrenal cortex is the thinnest layer found at the outer zone, covered by the capsule, consisted of closely packed groups of columnar or pyramidal cells forming arcades of cell columns. The cells contained many spherical mitochondria and well developed smooth surfaced endoplasmic reticulum but a compact Golgi apparatus in day 14 to month 1, 2, 6 (Fig. 1), and to month 12 (Fig. 3) and 24 animals. The zona fasciculata was the thickest layer, consisted of polygonal cells (Figs. 2,5) which were larger than the glomerulosa cells, arranged in long cords disposed radially to the medulla containing many lipid droplets (Figs. 2,5). At postnatal month 1, 2 and 6, the specific structure of 3 layers was completely developed and the arrangements of the cells in respective layer

became typical as adult tissues. By high power magnification at this stage, the cytoplasmic matrix can be observed full of numerous mitochondria and considerable number of lipid droplets. Observing the ultrastructure of the adrenocortical cells at the juvenile and adult stages, cell organelles including mitochondria were well developed from postnatal day 14 to month 1, month 2, month 6 (Figs. 1-3), month 12 (Figs. 4-6) and month 24. The mitochondria in the zona fasciculata (Fig. 2, 5) were less numerous and were more variable in size and shape than those of the glomerulosa cells (Figs. 1, 4), while the smooth surfaced endoplasmic reticulum were more developed and the Golgi apparatus was larger than the glomerulosa. In the zona reticularis (Figs. 3,6), the parallel arrangement of cell cords were anastomosed showing networks continued to the medullar cells. The mitochondria were less numerous and were more variable in size and shape than those of the glomerulosa cells like the fasciculata cells, as well as the smooth surfaced endoplasmic reticulum was developed and the Golgi apparatus was large like the fasciculata cells. Thus, the structure of the adrenocortical cells showed changes due to development and aging at respective developmental stages.

Radioautographic observations: Observing EM radioautograms, the silver grains were found over the nuclei of some adrenocortical cells labeled with ^3H -uridine, demonstrating RNA synthesis in all aging stages from perinatal stages at embryonic day 19, postnatal day 1 and day 3, day 9 and day 14 and adults at month 1, month 2, month 6 (Figs. 1-3), month 12 (Figs. 4-6) and month 24. Those labeled cells were found in all the 3 layers, the zona glomerulosa (Figs. 1,4), the zona fasciculata (Figs. 2,5) and the zona reticularis (Figs. 3, 6), at respective aging stages. In the labeled adrenocortical cells in 3 layers the silver grains were mainly localized over the euchromatin of the nuclei and nucleoli, or a few or several silver grains were found over cytoplasmic organelles such as endoplasmic reticulum, ribosomes and mitochondria showing RNA synthesis incorporating ^3H -uridine. The localizations of silver grains over the mitochondria were mainly on the mitochondrial matrices and some over the mitochondrial membranes when observed by high power magnification (Fig.7).

Quantitative analysis:

Number of mitochondria per cell: Preliminary quantitative analysis on the number of mitochondria

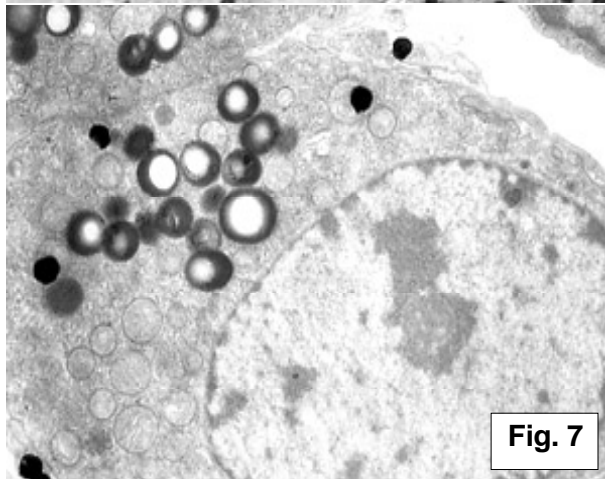
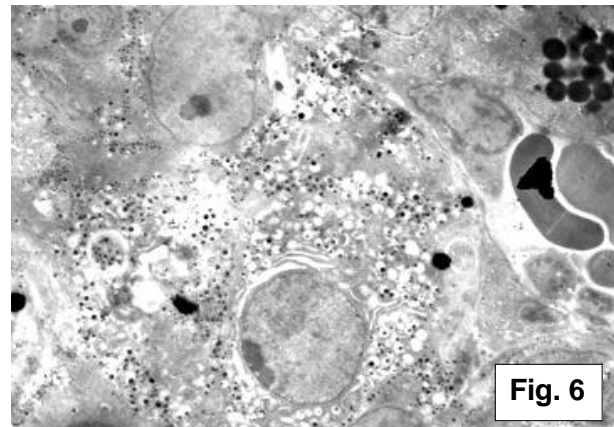
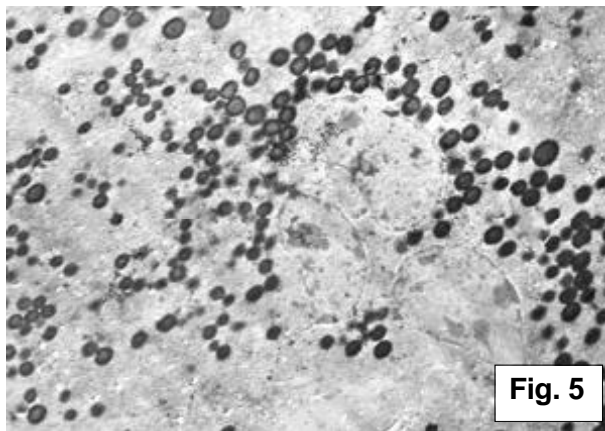
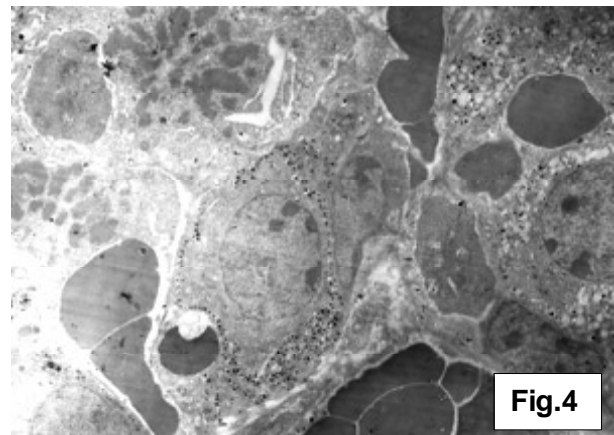
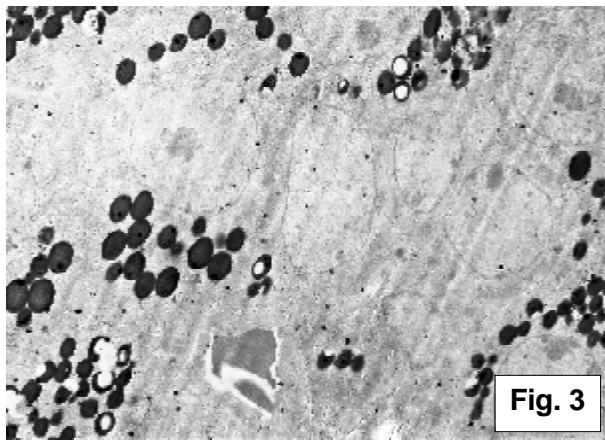
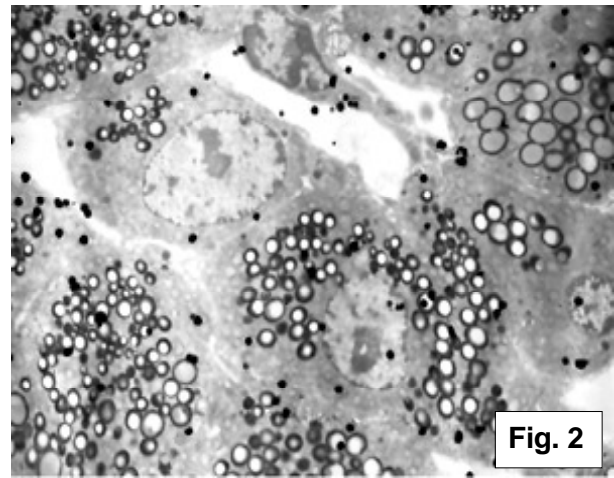
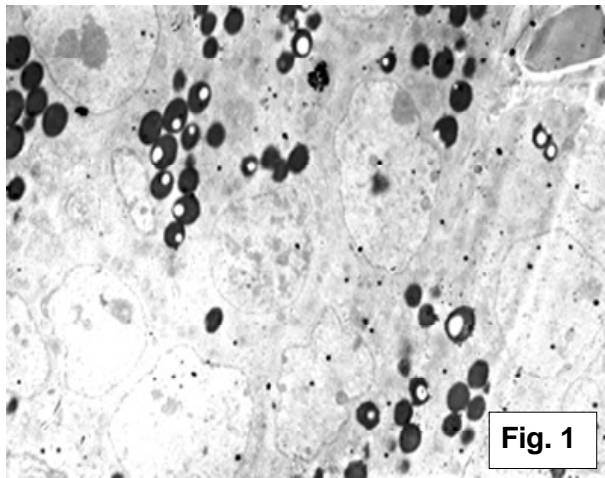


Fig. 1. Electron microscopic radioautogram of the zona glomerulosa of a young mouse aged at postnatal day 9, labeled with ^3H -uridine showing RNA synthesis with several silver grains in the nuclei as well as in a few mitochondria. x 4,000.

Fig. 2. Electron microscopic radioautogram of the zona fasciculata of a young mouse aged at postnatal day 9, labeled with ^3H -uridine showing RNA synthesis with several silver grains in the nuclei as well as in a few mitochondria. x 4,000.

Fig. 3. Electron microscopic radioautogram of the zona reticularis of a young mouse aged at postnatal month 9, labeled with ^3H -uridine showing RNA synthesis with several silver grains in the nuclei as well as a few mitochondria. x 4,000.

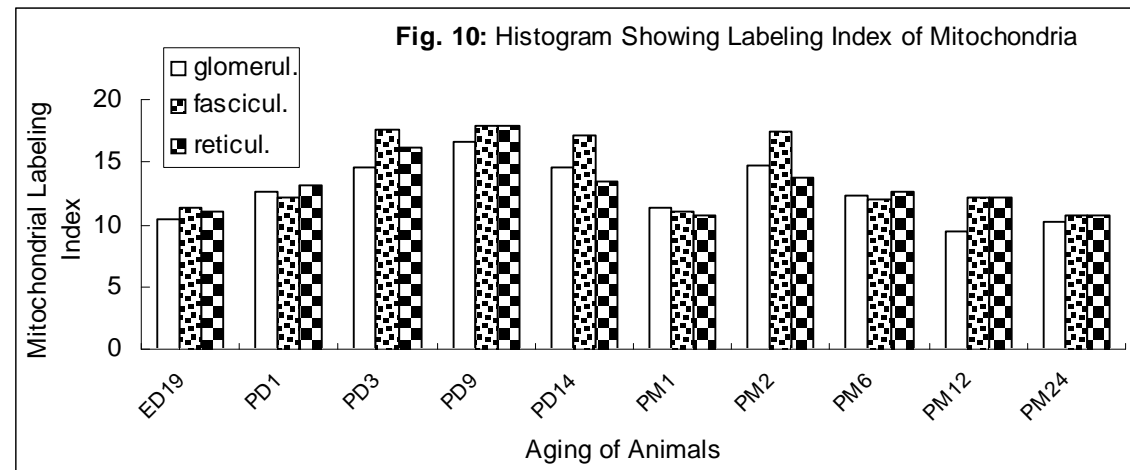
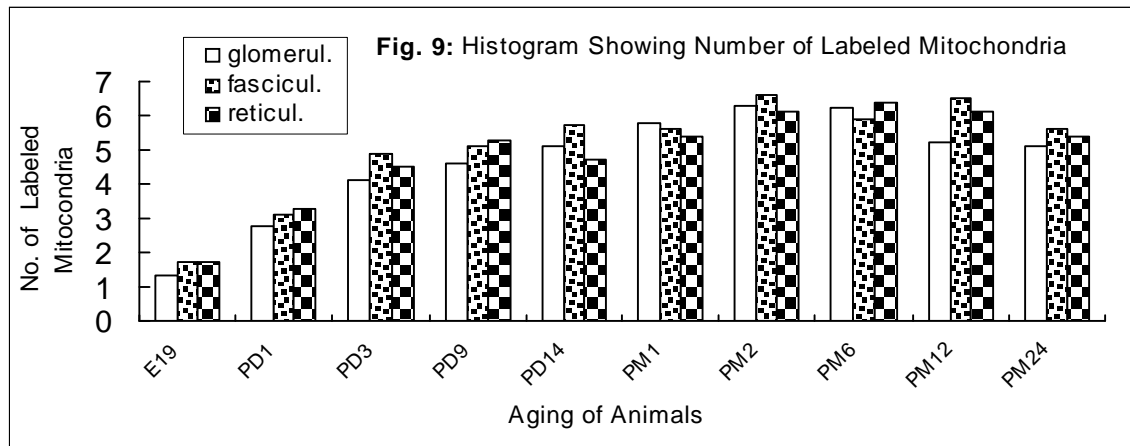
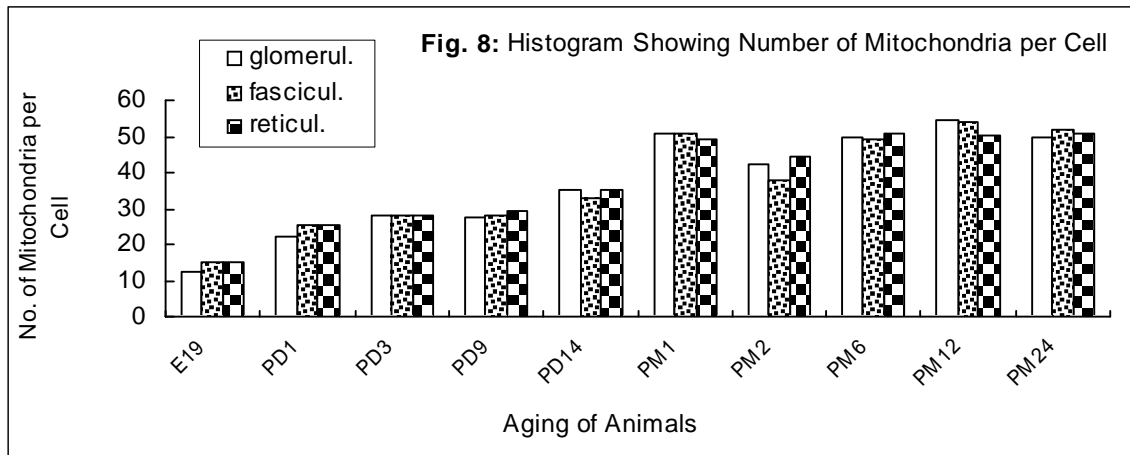


Fig. 4. Electron microscopic radioautogram of the zona glomerulosa of an old adult mouse aged at postnatal month 12, labeled with ^3H -uridine showing RNA synthesis with few silver grains in the nuclei as well as in a few mitochondria. $\times 4,000$.

Fig. 5. Electron microscopic radioautogram of the zona fasciculata of an old adult mouse aged at postnatal month 12, labeled with ^3H -uridine showing RNA synthesis with few silver grains in the nuclei as well as in a few mitochondria. $\times 4,000$.

Fig. 6. Electron microscopic radioautogram of the zona reticularis of an old adult mouse aged at postnatal month 12, labeled with ^3H -uridine showing RNA synthesis with few silver grains in the nuclei as well as in a few mitochondria. $\times 4,000$.

Fig. 7. High power magnification photograph of a part of Fig. 2, showing a few mitochondria are labeled with silver grains. $\times 8,000$.

Fig. 8. Histogram showing aging changes of the average numbers of mitochondria per cell in each adrenocortical cell in the 3 layers of respective animals in 10 aging groups.

Fig. 9. Histogram showing aging changes of the average numbers of labeled mitochondria with ^3H -uridine showing RNA synthesis per cell in each adrenocortical cell in the 3 layers of respective animals in 10 aging groups.

Fig. 10. Histogram showing aging changes of the average labeling index of mitochondria labeled with ^3H -uridine showing RNA synthesis per cell in each adrenocortical cell in the 3 layers of respective animals in 10 aging groups.

in 10 adrenocortical cells whose nuclei and cytoplasm were labeled with silver grains and other 10 cells whose nuclei and cytoplasm were not labeled in each aging group revealed that there was no significant difference between the number of mitochondria and the labeling indices ($P < 0.01$). Thus, the number of mitochondria and the labeling indices were calculated regardless whether their nuclei were labeled or not. The results obtained from the number of mitochondria in adrenocortical cells in the 3 layers of respective animals in 10 aging groups at perinatal stages, prenatal embryo day 19, postnatal day 1, 3, 9 and 14, showed an gradual increase from the prenatal day 19 (glomerulosa 12.5, fasciculata 14.5, reticularis 15.2/cell) to postnatal day 14 (glomerulosa 35.1, fasciculata 33.2, reticularis 35.3/cell), and to adult stages at postnatal month 1 (glomerulosa 50.7, fasciculata 50.8, reticularis 49.2/cell), then slightly decreased at month 2 (glomerulosa 42.4, fasciculata 37.6, reticularis 44.1/cell), but kept plateau from month 6 (glomerulosa 49.8, fasciculata 49.2, reticularis 50.6/cell), to month 12 (glomerulosa 54.7, fasciculata 53.8, reticularis 50.2/cell) and month 24 (glomerulosa 49.5, fasciculata 52.1, reticularis 50.6/cell), as is shown in the histogram (Fig. 8). The increase from embryo day 19 to postnatal month 1 was stochastically significant ($P < 0.01$).

Mitochondrial RNA synthesis: The results of visual grain counting on the number of mitochondria labeled with silver grains obtained from 10 adrenocortical cells in the 3 layers of each animal labeled with ^3H -uridine demonstrating RNA synthesis in 10 aging groups at perinatal stages, prenatal embryo day 19, postnatal day 1, 3, 9 and 14, month 1, 3, 6, 12 and 24, are plotted in Fig. 9. The results demonstrated that the numbers of labeled mitochondria with ^3H -uridine showing RNA synthesis per cell gradually increased from prenatal embryo day 19 (glomerulosa 1.3, fasciculata 1.7, reticularis 1.7/cell) to postnatal day 1 (glomerulosa 2.8, fasciculata 3.1, reticularis 3.3/cell), day 3 (glomerulosa 4.1, fasciculata 4.9, reticularis 5.5/cell), day 9 (glomerulosa 4.6, fasciculata 5.1, reticularis 5.3/cell), day 14 (glomerulosa 5.1, fasciculata 5.7, reticularis 4.7/cell), and month 1 (glomerulosa 5.8, fasciculata 5.6, reticularis 5.4/cell) and month 2 (glomerulosa 6.3, fasciculata 6.6, reticularis 6.1/cell), reaching the maximum, then decreased to month 6 (glomerulosa 6.2, fasciculata 5.9, reticularis 6.4/cell), month 12 (glomerulosa 5.2, fasciculata 6.5, reticularis 6.1/cell) and 24 (glomerulosa 5.1, fasciculata 5.6, reticularis

5.4/cell) as is shown in the histogram (Fig. 9).

The labeling index: On the other hand, the labeling indices in respective aging stages were calculated from the number of labeled mitochondria (Fig. 9) dividing by the number of total mitochondria per cell (Fig. 8), which were plotted in Fig. 10, respectively. The results showed that the labeling indices gradually increased from prenatal day 19 (glomerulosa 10.4, fasciculata 11.4, reticularis 11.1%) to postnatal newborn stage at postnatal day 1 (glomerulosa 12.6, fasciculata 12.1, reticularis 13.1%) and day 3 (glomerulosa 14.5, fasciculata 17.6, reticularis 19.6%), and to juvenile stage at postnatal day 9 (glomerulosa 16.6, fasciculata 18.0, reticularis 18.0%), reaching the maximum, and decreased to day 14 (glomerulosa 14.5, fasciculata 17.1, reticularis 13.4%) and to the adult stage at month 1 (glomerulosa 11.0, reticularis 10.7%) and month 2 (glomerulosa 10.0, fasciculata 11.4, reticularis 10.7%), to month 6 (glomerulosa 12.4, fasciculata 12.0, reticularis 12.6%) to month 12 (glomerulosa 9.5, fasciculata 12.1, reticularis 12.2%) and finally to senescence at month 24 (glomerulosa 10.3, fasciculata 10.7, reticularis 10.7%), as is shown in the histogram (Fig. 10).

DISCUSSION

From the results obtained at present, it was shown that intramitochondrial RNA synthesis was observed in adrenocortical cells in the 3 layers, zona glomerulosa, zona fasciculata and zona reticularis, of aging and senescent mice at various ages from prenatal embryos to postnatal newborn, juvenile and young adult to senescent stages until postnatal month 24 (year 2) and the number of mitochondria per cell showed increases due to aging, while the number of labeled mitochondria per cell and the labeling indices showed increases, reaching the maxima at postnatal day 9, and decreases due to aging and senescence. As for the macromolecular synthesis in various cells in various organs of experimental animals observed by light and electron microscopic radioautography, it is well known that the silver grains due to radiolabeled ^3H -thymidine demonstrate DNA synthesis [1, 41, 42], while the grains due to ^3H -uridine demonstrate RNA synthesis [15, 18-20].

The previous results obtained from the studies on the adrenocortical cells of aging mice by light microscopic radioautography revealed that silver grains indicating DNA synthesis incorporating ^3H -

thymidine were observed over the nuclei of some adrenocortical cells at perinatal stages from postnatal day 1 to 14 [32, 33]. However, they did not observe the intramitochondrial DNA synthesis. In the previous study [34], the numbers of silver grains showing nuclear DNA synthesis, as expressed by grain counting, did not give any significant difference between the cells in the 3 layers in the same aging groups. These results indicated that the amount of DNA synthesized in one nucleus was almost the same as in any other cells independent upon whether the nucleus belonged to any layers of the adrenal cortex. However, these differences between the 3 layers at respective aging groups were not stochastically significant ($P < 0.01$). These results indicated that the DNA synthetic activity in the nuclei of 3 layers of the adrenal cortex did not show any difference. To the contrary, the numbers of mitochondria labeled with ^3H -thymidine per cell as well as the labeling indices between the aging groups increased gradually from prenatal embryo day 19, to postnatal day 1, 3, 9 and 14, to postnatal month 1 and 2, then decreased to month 6, 12 and 24. These decreases were stochastically significant ($P < 0.01$). These results indicated that the mitochondria in adrenocortical cells proliferated from perinatal and postnatal newborn stage to adult stage at postnatal month 1 and 2, reaching the maxima, then lost their proliferating activities from aged stage at month 6 to senescent stage up to month 24.

On the other hand, the radioautograms in the present study showing incorporations of ^3H -uridine into mitochondria indicating mitochondrial RNA synthesis resulted in silver grain localization over the mitochondria independently from the nuclei whether the nuclei were labeled with silver grains or not in almost all the cells in the 3 layers of the adrenocortical cells from prenatal embryo day 19 to postnatal day 1, 3, 9 and 14, to postnatal month 1, 2, 6, 12 and 24, during the development and aging. The numbers of labeled mitochondria showing RNA synthesis increased from perinatal day to postnatal adult stage at month 2, then kept plateau, while the labeled mitochondria with ^3H -uridine showing RNA synthesis increased from perinatal stage to postnatal adult stage at month 2, then decreased at month 24. As the results, the labeling indices increased from perinatal embryonic day to postnatal newborn and juvenile stages at day 3, 9 and 14, then decreased from day 14 to month 1 and again increased at month 2 and finally decreased to senescence at month 24. These

changes demonstrate the aging changes. The results obtained previously [34] indicated that mitochondria in the adrenocortical cells proliferated from newborn to adult stages around month 1 and 2, showing mitochondrial DNA synthesis, while the present results indicate that mitochondrial RNA synthesis increased from newborn stage to postnatal day 3, 9 and 14, then decreased from day 14, to month 1, but increased at month 2 and again decreased to month 24. These results showed that the RNA synthetic activity changed due to aging of individual animals. As for the relationship between the mitochondrial RNA synthesis and the aging and senescence of individual animals, mitochondrial RNA may be relevant to physiological functions of mitochondria such cellular metabolic activity as the secretion of hormones regulating carbohydrate metabolims and fluid-salt balance, adjusting them to the needs of growing and senescent individual mice in aging.

Thus, the results obtained from the adrenal glands of aging mice at present should form a part of special cytochemistry [41], as well as a part of special radioautography [42], i.e., the application of radioautography to the adrenal glands, as was formerly reviewed by the present author. We expect that such special radioautography and special cytochemistry should be further developed in all the organs of various animals in the future.

CONCLUSIONS

From the results obtained at present, it was concluded that almost all the cells in the 3 layers of the adrenal cortex of mice at various ages, from prenatal embryo day 19 to postnatal newborn, day 1, 3, 9 and 14, and to postnatal month 1, 2, 6, 12 and 24, were labeled with silver grains showing RNA synthesis with ^3H -uridine in their mitochondria. Quantitative analysis on the number of mitochondria in adrenocortical cells in the 3 layers resulted in an increase from the prenatal day to postnatal day 1, 3, 9, 14, and month 1 and 2, and 6, reaching the maximum at postnatal month 12, then a little decreased to month 24. To the contrary, the numbers of labeled mitochondria with ^3H -uridine showing RNA synthesis increased from perinatal stage to postnatal juvenile stage at day 9, and decreased to aging and senescence, while the mitochondrial labeling index also increased from prenatal day to postnatal day 1, 3 and 9, reaching the maximum at postnatal day 9, then decreased to day 14, month 12 and 24 due to aging. These results

demonstrated that the number of mitochondria in adrenal cortical cells increased by proliferating themselves synthesizing mitochondrial DNA and RNA at perinatal stages to postnatal month 1, 2, and 6 due to aging of animals, while the activity of mitochondrial DNA and RNA syntheses increased and decreased from perinatal stage to senescence at month 12 or 24 due to aging and senescence.

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