Electron Microscopic Radioautographic Study on Mitochondrial DNA and RNA Syntheses in Pulmonary Cells of Aging Mice

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ABSTRACT

In order to study the aging changes of intramitochondrial DNA and RNA syntheses in the lungs of mice, the pulmonary tissues were obtained from 11 small groups of normal ddY strain mice, each consisting of 3 litter mates of both sexes, total 33, aged from embryonic day 6, 8 to postnatal day 1, 3, 7 and 14 and month 1, 2, 6, 12 and 22, which were cut into small tissue blocks and incubated in Eagle’s MEM containing either ³H-thymidine or ³H-uridine in vitro in a CO₂ incubator for 1 hr., fixed with cold buffered 2.5% glutaraldehyde at pH 7.4 for and postfixed in 1% osmium tetroxide at 4°C, dehydrated, embedded in epoxy resin and were processed for electron microscopic radioautography. On electron microscopic radioautograms obtained from each animal, the number of mitochondria, the number of labeled mitochondria and the mitochondrial labeling index labeled with ³H-thymidine or ³H-uridine showing DNA or RNA syntheses in each pulmonary cells, type I and II epithelial cells, interstitial cells and endothelial cells, were counted and the averages in respective aging groups were compared. From the results, it was demonstrated that the numbers of mitochondria, the numbers of labeled mitochondria and the labeling indices of intramitochondrial DNA and RNA syntheses in respective cells of mice at various ages increased and decreased according to aging of animals.

KEY WORDS:  mitochondria, EM radioautography, DNA & RNA syntheses, mouse pulmonary cells

INTRODUCTION

Intramitochondrial nucleic acid syntheses, both DNA and RNA, in mammalian and avian cells were first demonstrated morphologically by the present authors by means of electron microscopic radioautography with accurate localization in primary cultured cells of the liver and kidney of mice and chickens in vitro (Nagata et al., 1967a, 1967b) and then in some other established cell lines such as HeLa cells (Nagata 1972a,b,c,d) or mitochondrial fractions prepared from in vivo cells (Nagata 1974, Nagata et al., 1975, 1976). It was later commonly found in various cells and tissues not only in vitro obtained from various organs in vivo (Nagata 1984, Nagata and Murata 1977, Nagata et al., 1977 a,b,c), but also in vivo cells of various organs such as the salivary glands (Nagata et al., 2000), the liver (Nagata et al., 1979, 1982a,b, Ma and Nagata 1988a,b, Ma et al., 1994, Nagata 2005, 2006, Nagata and Ma 2005a,b), the pancreas (Nagata 1992, Nagata et al., 1986), the trachea (Sun 1995, Sun et al., 1994, 1995, 1997), the kidney (Hanai and Nagata 1994), the testis (Gao et al., 1994, 1995), the uterus (Yamada and Nagata 1994a,b), the adrenal (Ito and Nagata 1996, Liang et al., 1999), the brain (Cui et al., 1996), the retina (Gunarso 1984, Gunarso et al., 1996, 1997, Kong and Nagata 1994) of mice, rats and chickens. The relationship
between the intramitochondrial RNA synthesis and cell cycle was formerly studied and it was clarified that the intramitochondrial DNA synthesis was performed without nuclear involvement (Nagata 1972b). However, the relationship between the DNA and RNA synthesis and the aging of individual animals has recently been studied in the liver of aging mice (Nagata 2005, 2006). This paper deals with the relationship between the DNA and RNA syntheses and aging in the pulmonary cells of mice in vivo at various ages by means of electron microscopic radioautography as a part of serial studies on special cytochemistry (Nagata 2001) and radioautographology (Nagata 2002).

MATERIALS AND METHODS

Animals

The pulmonary tissues were obtained from 11 small groups of normal ddY strain mice, each consisting of 3 litter mates of both sexes, total 33, aged from embryonic day 16, 18 to postnatal day 1, 3, 7 and 14 and month 1, 2, 6, 12 and 22. 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. The mean life span of the experimental animals we used, ddY strain mice, which were bred in our laboratory under above conditions, was around 20 months in average. Thus, we used the term senescent stage as the animals over 12 months to 22 months. All the experiments treating animals in this study were carried out at Department of Anatomy and Cell Biology, Shinshu University School of Medicine, Matsumoto, Japan, and the procedures concerning the animal experiments were in accordance with the guidelines of the animal research committee of Shinshu University School of Medicine as well as the principles of laboratory animal care (NIH publication No. 86-23, revised 1985).

Tissue Processing

After the experimental animals were sacrificed by decapitation under Nembutal (Abbott Laboratories, Chicago, ILL, USA) anesthesia, the lung tissues from the right inferior lobes were taken out aseptically and were cut into small tissue blocks (1mm x 1mm x 1mm) in Hanks solution (Nissui, Tokyo, Japan) in a sterile room in Tissue Culture Laboratory. The tissue blocks were washed in Hanks solution and incubated in Eagle’s MEM (Nissui, Tokyo, Japan) containing 10 fetal bovine serum (GIBCO, Grand Island, N.Y., USA) and either \(^3\)H-thymidine (Amersham, England, specific activity 877 GBq/mM) or \(^3\)H-uridine (Amersham, England, 1.11 TBq/mM) at concentrations of 3.7 MBq/ml medium) in vitro in a CO\(_2\) incubator (Tabai, Tokyo, Japan) with 5% CO\(_2\) in air. After the in vitro incubation for 1 hr., the tissue blocks were washed 3 times in Hanks solution, fixed with cold 2.5% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4 for 1 hr. and postfixied 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).

Radioautography

Thick sections at 1 µm thickness from respective specimens were cut in sequence on a Porter-Blum MT-2B ultramicrotome (Dupont-Sorvall, Newtown, MA, USA) using glass knives and collected on glass slides, coated with Konica NR-M2 radioautographic emulsion (Konica, Tokyo, Japan) by a dipping method (Nagata 1992, 1997, 2002) for light microscopy, exposed for 4 weeks at 4°C, developed in SDX-1 developer at 20°C for 5 min, fixed in acid fixer, stained in 0.1% toluidine blue/0.1M sodium phosphate (pH7.4) solution. On the other hand, in order to shorten the
exposure time for electron microscopic radioautography, semithin sections at 0.2µm thickness were cut in sequence on the same ultramicrotome and 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 (Nagata 1992, 1996, 1997, 2002), exposed for 10 months at 4°C, developed in phenidon developer at 16°C for 1 min after 30 sec gold-latensification in freshly prepared gold thiocyanate solution and stained with lead citrate solution for 3 min (Nagata 1992, 1996, 1997, 2002).

The light microscopic radioautograms were examined and photographed with an Olympus Vanox AHB-LB light microscope (Olympus, Tokyo, Japan), while the electron microscopic radioautograms were examined in either a Hitachi H-700 electron microscope (Hitachi, Tokyo, Japan) at accelerating voltage of 00 kV or a JEOL JEM-4000EX electron microscope (JEOL, Tokyo, Japan) at accelerating voltage of 400 kV for observing thicker specimens.

**Quantitative Analysis of Light Microscopic Radioautograms**

On light microscopic radioautograms obtained from the specimens labeled with \(^{3}\)H-thymidine, 3 specimens from each animal were observed for analysis in order to calculate the labeling indices of respective pulmonary cell types. From each slides at least 300 cells and altogether 1000 cells in the alveolar walls of the lung of each animal were counted randomly under an oil immersion 100x objective lens and the numbers of labeled cells with silver grains due to \(^{3}\)H-thymidine showing DNA synthesis were recorded. A pulmonary cell was considered labeled if 5 or more silver grains were observed over the nucleus, because the background fogs over the same area size of respective nuclei were less than 2. The labeling index of each cell type in the pulmonary cells, including type I epithelial cells, type II epithelial cells, interstitial cells and endothelial cells, at each time interval was calculated by counting at least 3000 cells in each age group. The labeling indices in respective aging groups were calculated with a personal computer and were expressed as mean ± standard deviation. The stochastical analysis was performed by Student’s t test for the difference between respective age groups and the results were considered to be significant at P<0.05.

On light microscopic radioautograms obtained from the specimens labeled with \(^{3}\)H-uridine, on the other hand, 3 specimens from each animal were observed for analysis in order to calculate the grain counts of respective 4 pulmonary cell types. From each slides at least 300 cells and altogether 1000 cells in the alveolar walls of the lung of each animal were counted randomly under an oil immersion 100x objective lens and the numbers of silver grains due to \(^{3}\)H-uridine showing RNA synthesis over both the nuclei and cell bodies were recorded separately.

The labeling indices in respective aging groups labeled with \(^{3}\)H-thymidine and the grain counts over the nucleus and cell body in respective aging groups labeled with \(^{3}\)H-uridine were calculated with a personal computer (Macintosh type 8100/100, Apple Computer, Tokyo, Japan) and were expressed as mean ± standard deviation. The stochastical analysis was performed by Student’s t test for the difference between respective age groups and the results were considered to be significant at P<0.05.

**Quantitative Analysis of Electron Microscopic Radioautograms**

In order to analyze the electron microscopic radioautograms obtained from the specimens either labeled with \(^{3}\)H-thymidine or \(^{3}\)H-uridine, 20 electron radioautograms showing cross sections of pulmonary cells including nuclei from each group, based on the electron microscopic photographs taken after observation on 100 pulmonary cells at least from respective animals, were analyzed to calculate the number of mitochondria, number of labeled mitochondria with silver
grains by visual counting. In order to estimate the background fog, the number of silver grains in respective radioautograms in 10 circles with the same area size as a mitochondrion selected at random on the plastic sections outside of cell body coated with radioautographic emulsions were counted. The average number of silver grains per mitochondrial area was 0.01-0.03/area in respective groups. Then, we considered that almost no chemographic effects of pulmonary sections as well as radioautographic procedures were detected. Therefore, the grain count in each specimen was not corrected. Thus, the mitochondrion which was labeled with more than one silver grains was defined as labeled. From all the data thus obtained the averages and standard deviations in respective aging groups were computed with a personal computer (Macintosh type 8100/100, Apple Computer, Tokyo, Japan). The data were stochastically analyzed using variance and Student’s t-test. The differences were considered to be significant at P value <0.01.

RESULTS

Morphological Observations

The pulmonary tissues obtained from ddY strain mice at various ages consisted of several kinds of cells, the type I epithelial cell or the small alveolar epithelial cell, the type II epithelial cell or the large alveolar epithelial cell, the interstitial cell, the endothelial cell and alveolar phagocyte or dust cell as we had formerly observed (Sun 1995, Sun et al., 1994, 1995, 1997). At embryonic day 6 and 8, the fetal lung tissues appeared as glandular organizations consisting of many alveoli bordering undifferentiated cuboidal cells and no squamous epithelial cells were seen (Figure E). Mitotic figures were frequently observed in cuboidal epithelial cells. After birth, the structure of the alveoli was characterized by further development of the alveolar-capillary networks from postnatal day 1 to 3 and 7 (Figure F). During the development, the cellular composition of the alveolar epithelium resembled that of the adult lung, with a mixed population of the type I and type II epithelial cells. Up to 1 and 2 weeks after birth, the lung tissues showed complete alveolar structure and single capillary system almost the same as the adult after 1 month to 2 to 6 months (Figures A, B), and further to senescent stage over 12 months (Figure C) to 22 months (Figure D).

Quantitative Analysis of Light Microscopic Radioautograms

Observing the labeled cells with $^3$H-thymidine showing DNA synthesis, many cuboidal epithelial cells, interstitial cells and endothelial cells were predominantly labeled with silver grains corresponding to S-phase of the cell cycle (Figure 1). The numbers of labeled cells with $^3$H-thymidine gradually decreased from fetal stage (Figure 1E) to postnatal day 1, 3, 7 (Figure 1F) and 14. After 1, 2 and 6 months at adult stage, only a few labeled cells were found (Figures 1A, 1B). After 12 and 22 months at senescent stage, only a few of type II epithelial cells (Figure 1D) and interstitial cell (Figure 1C) were labeled. The labeling indices of respective types of pulmonary cells as expressed by mean ± standard deviation are shown in Figure 2. The labeling index of the type I epithelial cells was very low at fetal stage, reached the maximum on the postnatal day 3 (7.9%) and decreased gradually with aging, fell down to 0% at 6 months (Figure 2A). The labeling index of the type II epithelial cells, on the other hand, was very high at fetal stage, reached the maximum on embryonic day 16 (47.1%), declined on embryonic day 18 (17.2%), and gradually decreased with aging to 0.33% at 6 months and 0.42% at 22 months but never dipped to 0% (Figure 2B). The labeling index of the interstitial cells was the highest on embryonic day 16 (38.1%) and decreased due to aging dropping to 0% at 22 months (Figure 2C). The labeling index of the endothelial cells was also the highest on embryonic day 16 (29.2%), decreased rapidly on
Figure 1. Light microscopic radioautograms of the pulmonary tissues of ddY mice labeled with $^3$H-thymidine showing DNA synthesis.

Figure 1A. Light microscopic radioautogram of the pulmonary tissue from a mouse at postnatal 6 months. A few labeled interstitial cells are observed.

Figure 1B. Light microscopic radioautogram of the pulmonary tissue of a mouse at postnatal 6 months. A labeled type II epithelial cell (arrow) can be seen.

Figure 1C. Light microscopic radioautogram of the pulmonary tissue of a mouse at postnatal 12 months. A labeled type II epithelial cell (arrow) can be seen.

Figure 1D. Light microscopic radioautogram of the pulmonary tissue of a mouse at postnatal 22 months. No labeled cell can be seen.

Figure 1E. Light microscopic radioautogram of the pulmonary tissue of a mouse embryo at fetal day 16. Many undifferentiated cell are labeled.

Figure 1F. Light microscopic radioautogram of the pulmonary tissue of a mouse at postnatal day 14 (2 weeks). A few labeled cells can be seen.
embryonic day 18 (17.6%), reaching 0% at 22 months (Figure 2D).

The light microscopic radioautograms labeled with $^3$H-uridine showing RNA synthesis, on the other hand, almost all the cuboidal epithelial cells, interstitial cells and endothelial cells were labeled with silver grains over both the nuclei and cell bodies. The alveoli of the embryos on day 16 appeared glandular organization lined exclusively with undifferentiated type II cuboidal epithelial cells. On the postnatal day 1, 3 and 7, the alveolar epithelium consisted of both types of epithelial cells, type I and type II, incorporating many silver grains over both the nuclei and cell bodies. From postnatal 2 weeks to 6 months after birth, many silver grains were observed over the nuclei and cytoplasm of the type I, type II epithelial cells, interstitial cells and endothelial cells. From postnatal month 12 to 22 at senescent stage, only a few silver grains were seen over the nuclei and cytoplasm of all the cell types. The grain counts of 4 cell types at various aging stages from embryonic day 16 to postnatal month 22 were depicted in Figures 3A, 3B, 3C and 3D.

Figure 2. Histograms showing labeling indices of respective types of pulmonary cells of ddY mice at various ages from fetal day 16 to postnatal month 22 labeled with $^3$H-thymidine showing DNA synthesis.

Figure 2A. Histogram showing labeling indices of the type I epithelial cells.
Figure 2B. Histogram showing labeling indices of the type II epithelial cells.
Figure 2C. Histogram showing labeling indices of the interstitial cells.
Figure 2D. Histogram showing labeling indices of the endothelial cells.
Quantitative Analysis of Electron Microscopic Radioautograms

Mitochondrial DNA synthesis

On electron microscopic radioautograms labeled with $^3$H-thymidine, silver grains were observed over the nuclei of some pulmonary cells corresponding to the DNA synthesis in S-phase as observed by light microscopic radioautography. On the other hand, some mitochondria in both S-phase cells and interphase cells which did not show any silver grains over their nuclei were labeled with silver grains showing intramitochondrial DNA synthesis. The intramitochondrial DNA synthesis was observed in all the cell types, the type I epithelial cell (Figure 4), the type II epithelial cell (Figure 5), the interstitial cell (Figure 6) and the endothelial cell. Because enough numbers of electron photographs (more than 5) were not obtained from all the cell types in respective aging groups, only some cell types and some aging groups where enough numbers of electron photographs were available were used for quantitative analysis. The numbers of mitochondria per cell profile area, the numbers of labeled mitochondria per cell and the labeling indices of the type I epithelial cells in only a few aging groups was shown in Figure 7. Likewise, the similar results from the type II epithelial cells (Figure 8), the interstitial cells (Figure 9) were shown. The labeling indices in

Figure 3. Histograms showing silver grain counts in both nuclei and cytoplasm of respective types of pulmonary cells of ddY mice at various ages from fetal day 16 to postnatal month 22 labeled with $^3$H-uridine showing RNA synthesis.

Figure 3A. Histogram showing silver grain counts of the type I epithelial cells.
Figure 2B. Histogram showing silver grain counts of the type II epithelial cells.
Figure 2C. Histogram showing silver grain counts of the interstitial cells.
Figure 2D. Histogram showing silver grain counts of the endothelial cells.
Figure 4. Electron microscopic radioautogram of the pulmonary type I epithelial cells of a fetal day 18 mouse embryo labeled with $^3$H-thymidine showing DNA synthesis. Several silver grains are localized over two nuclei of 2 cells showing nuclear DNA synthesis incorporating $^3$H-thymidine. On the other hand, a few silver grains are localized over a few mitochondria of these cells showing mitochondrial DNA synthesis. x 10,000.

Figure 5. Electron microscopic radioautogram of the pulmonary type II epithelial cells of a fetal day 18 mouse embryo labeled with $^3$H-thymidine showing DNA synthesis. Several silver grains are localized over two nuclei of 2 cells showing nuclear DNA synthesis. On the other hand, a few silver grains are localized over a few mitochondria of these cells showing mitochondrial DNA synthesis. x 10,000.
Figure 6. Electron microscopic radioautogram of the pulmonary interstitial cells of a fetal day 18 mouse embryo labeled with $^{3}$H-thymidine showing DNA synthesis. Several silver grains can be seen over a nucleus at left showing nuclear DNA synthesis. On the other hand, several silver grains can be seen over a few mitochondria showing mitochondrial DNA synthesis. x 10,000.

Figure 7. Histograms demonstrating aging changes of mitochondrial DNA synthesis in the pulmonary type I epithelial cells labeled with $^{3}$H-thymidine.

Bottom: The averages of the total number of mitochondria per cellular profile area in the pulmonary type I epithelial cells at respective aging groups from embryonic day 16 to postnatal month 22.

Middle: The averages of the total number of mitochondria labeled with $^{3}$H-thymidine showing DNA synthesis per cellular profile area in the pulmonary type I epithelial cells at respective aging groups from embryonic day 16 to postnatal month 22.

Top: The averages of the labeling index of mitochondria labeled with $^{3}$H-thymidine showing DNA synthesis (number of labeled mitochondria / number of total mitochondria) in the pulmonary type I epithelial cells at respective aging groups.
respective aging stages were calculated from the number of labeled mitochondria and the number of total mitochondria per cellular profile area which were plotted in Figures 7-9, respectively. These results demonstrated that the labeling indices in these cell types decreased from perinatal stages to the adult and senescent stage due to aging.

**Mitochondrial RNA synthesis**

On electron microscopic radioautograms labeled with $^3$H-uridine, silver grains were observed over the nuclei of some pulmonary cells corresponding to the RNA synthesis in most cells in cell cycle in respective aging groups as observed by light microscopic radioautography. The silver grains were observed to localize not only over euchromatin and nucleoli in the nuclei but also over many cell organelles such as endoplasmic reticulum, ribosomes, and mitochondria as well as cytoplasmic matrices of all the cell types. The intramitochondrial RNA synthesis was observed in all the cell types, the type I epithelial cell (Figure 10), the type II epithelial cell (Figure 11), the interstitial cell (Figure 12) and the endothelial cell. Because enough numbers of electron photographs (more than 5) were not obtained from all the cell types in respective aging groups, only some cell types and some aging groups where enough numbers of electron photographs were available were used for quantitative analysis similarly to DNA synthesis. The numbers of mitochondria per cell profile area, the numbers of labeled mitochondria per cell and the labeling indices of the type I epithelial cells in only a few aging groups was shown in Figure 13. Likewise, the similar results from the type II epithelial cells (Figure 14), the interstitial cells (Figure 15), and the endothelial cell (Figure 16) were shown. The labeling indices in respective aging stages were
calculated from the number of labeled mitochondria and the number of total mitochondria per cellular profile area which were plotted in Figures 13-16, respectively. These results demonstrated that the numbers of labeled mitochondria in these cell types increased from perinatal stages to the adult stage, reaching maxima at postnatal month 1, and decreased to the senescent stage due to aging.

**DISCUSSION**

From the results obtained in the present study, it was shown that both intramitochondrial DNA and RNA syntheses were observed in 4 cell types of pulmonary tissues, the type I epithelial cells, the type II epithelial cells, the interstitial cells, and the endothelial cells of aging mice at various ages from prenatal embryos to postnatal newborn, young, adult and senescent stages and the number of mitochondria per cell, the number of labeled mitochondria and the labeling indices showed increases and decreases due to aging, respectively. Concerning to 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 the radiolabeled precursor

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**figure 9.** Histograms demonstrating aging changes of mitochondrial DNA synthesis in the pulmonary interstitial cells labeled with $^3$H-thymidine.

Bottom: The averages of the total number of mitochondria per cellular profile area in the pulmonary interstitial cells at respective aging groups from embryonic day 16 to postnatal month 22.

Middle: The averages of the total number of mitochondria labeled with $^3$H-thymidine showing DNA synthesis per cellular profile area in the pulmonary interstitial cells at respective aging groups from embryonic day 16 to postnatal month 22.

Top: The averages of the labeling index of mitochondria labeled with $^3$H-thymidine showing DNA synthesis (number of labeled mitochondria / number of total mitochondria) in the pulmonary interstitial cells at respective aging groups.

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Figure 0. Electron microscopic radioautogram of the pulmonary type I epithelial cell of a postnatal month 1 mouse labeled with $^3$H-uridine showing RNA synthesis. Several silver grains are localized over the nucleus of the cell showing nuclear RNA synthesis incorporating $^3$H-uridine. On the other hand, a few silver grains are localized over a few mitochondria of the cell showing mitochondrial RNA synthesis. $x$ 10,000.

Figure 11. Electron microscopic radioautogram of the pulmonary type II epithelial cell of a postnatal month 1 mouse labeled with $^3$H-uridine showing RNA synthesis. Several silver grains are localized over the nucleolus and chromatin in the nucleus of the cell showing nuclear RNA synthesis. On the other hand, a few silver grains are localized over a few mitochondria of these cells showing mitochondrial RNA synthesis. $x$ 10,000.
Figure 12. Electron microscopic radioautogram of the pulmonary interstitial cells of a postnatal month 1 mouse labeled with $^3$H-uridine showing RNA synthesis. Several silver grains can be seen over the nucleus at center showing nuclear RNA synthesis. On the other hand, several silver grains can be seen over a few mitochondria showing mitochondrial RNA synthesis. x 10,000.

Figure 13. Histograms demonstrating aging changes of mitochondrial RNA synthesis in the pulmonary type I epithelial cells labeled with $^3$H-uridine.

Bottom: The averages of the total number of mitochondria per cellular profile area in the pulmonary type I epithelial cells at respective aging groups from embryonic day 16 to postnatal month 22.

Middle: The averages of the total number of mitochondria labeled with $^3$H-uridine showing RNA synthesis per cellular profile area in the pulmonary type I epithelial cells at respective aging groups from embryonic day 16 to postnatal month 22.

Top: The averages of the labeling index of mitochondria labeled with $^3$H-uridine showing DNA synthesis (number of labeled mitochondria / number of total mitochondria) in the pulmonary type I epithelial cells at respective aging groups.
Bottom: The averages of the total number of mitochondria per cellular profile area in the pulmonary type II epithelial cells at respective aging groups from embryonic day 6 to postnatal month 22.

Middle: The averages of the total number of mitochondria labeled with $^3$H-uridine showing RNA synthesis per cellular profile area in the pulmonary type II epithelial cells at respective aging groups from embryonic day 16 to postnatal month 22.

Top: The averages of the labeling index of mitochondria labeled with $^3$H-uridine showing DNA synthesis (number of labeled mitochondria / number of total mitochondria) in the pulmonary type II epithelial cells at respective aging groups.

Figure 14. Histograms demonstrating aging changes of mitochondrial RNA synthesis in the pulmonary type II epithelial cells labeled with $^3$H-uridine.

Bottom: The averages of the total number of mitochondria per cellular profile area in the pulmonary interstitial cells at respective aging groups from embryonic day 16 to postnatal month 22.

Middle: The averages of the total number of mitochondria labeled with $^3$H-uridine showing RNA synthesis per cellular profile area in the pulmonary interstitial cells at respective aging groups from embryonic day 16 to postnatal month 22.

Top: The averages of the labeling index of mitochondria labeled with $^3$H-uridine showing DNA synthesis (number of labeled mitochondria / number of total mitochondria) in the pulmonary interstitial cells at respective aging groups.

Figure 15. Histograms demonstrating aging changes of mitochondrial RNA synthesis in the pulmonary interstitial cells labeled with $^3$H-uridine.
such as $^3$H-thymidine and $^3$H-uridine demonstrate DNA and RNA syntheses, respectively (Nagata et al., 1967a,b, Nagata 1972a,b, 1992, 1996, 1997, 2002). The present results obtained from the lungs of aging mice revealed that the incorporation of $^3$H-thymidine and $^3$H-uridine indicating DNA and RNA syntheses resulted in silver grain localization over the nuclei and cell bodies of 4 cell types of pulmonary cells from perinatal animals at embryonic day 19, postnatal day 1, 2, 9, 14 to adult and senescent stages at postnatal month 1, 2, 6, 12 and 24, which showed the localization of newly synthesized DNA and RNA in the nuclei and the cytoplasmic cell organelles including mitochondria. From the results obtained in the present study, the numbers of mitochondria in respective pulmonary cells showed increases and decreases, while the numbers of labeled mitochondria with silver grains due to $^3$H-thymidine and $^3$H-uridine incorporation demonstrating newly synthesized DNA and RNA also showed increases and decreases reaching the maximum at postnatal month 1, and the labeling indices of mitochondria labeled with $^3$H-thymidine and $^3$H-uridine showed increases and decreases reaching the maximum at postnatal month 1.

thymidine or \(^{3}H\)-uridine were observed not only over the nuclei of some hepatocytes but also over the mitochondria showing intramitochondrial DNA and RNA synthesis (Nagata and Ma, 2003a,b; 2005a,b).

With regards to the incorporations of \(^{3}H\)-thymidine or \(^{3}H\)-uridine into mitochondria demonstrating DNA or RNA syntheses, many authors previously reported that DNA synthesis was observed by means of electron microscopic radioautography in lower organism such as slime mold (Guttes and Guttes 1964, Schuster 1965), tetrahymena (Stone and Miller 1965) or chicken fibroblasts in tissue culture under abnormal conditions (Chêvremont 1963, Meyer 1966) or liver and kidney cells of chicken and mouse under normal conditions (Nagata et al., 1969) as was formerly reviewed (Schatz, 1970; Tandler and Hoppel, 1972). Likewise, RNA synthesis in Agaricus (Vogel and Kemper, 1967), Ochromonas (Gibbs, 1968), chicken liver (André, 1968; André and Marini, 1965), mouse liver (Curgy, 1968; 1970), rat adrenal cortex (Nussdorfer and Mazzochi, 1971) and human cells (Nagata, 1969, 1972a,b,c, 1974) were also demonstrated.

On the other hand, the incorporations of \(^{3}H\)-thymidine into mitochondria demonstrating DNA synthesis were formerly observed by means of electron microscopic radioautography not only in lower organisms such as slime mold (Guttes and Guttes 1964, Schuster 1965), tetrahymena (Stone and Miller 1965; Chi and Suyama, 1970) but also in higher animals such as chicken fibroblasts in tissue culture under abnormal conditions (Chêvremont 1963, Meyer 1966) or liver and kidney cells of chicken and mouse under normal conditions (Nagata et al., 1969). Then we also demonstrated intramitochondrial DNA synthesis incorporating \(^{3}H\)-thymidine or RNA synthesis incorporating \(^{3}H\)-uridine in some other established cell lines originated from human being such as HeLa cells (Nagata 1972a,b,c) or mitochondrial fractions prepared from in vivo mammalian cells such as rat and mouse (Nagata 1974, Nagata et al., 1975, 1976). It was later commonly found in various cells and tissues in vitro obtained from various organs such as the cultured human uterus cancer cells HeLa (Nagata 1984, Nagata et al., 1977b), cultured rat sarcoma cells (Nagata et al., 1977a), mouse liver and pancreas cells in vitro (Nagata and Murata 1977, Nagata et al., 1977c), but also in vivo cells obtained from various organs such as the salivary gland (Nagata et al., 2000), the liver (Nagata et al., 1979, 1982a,b, Ma and Nagata 1988a,b; 1990; Ma et al., 1994), the pancreas (Nagata 1992, Nagata et al., 1986), the trachea (Sun et al., 1997), the kidney (Hanai and Nagata 1994), the testis (Gao et al., 1994, 1995), the uterus (Yamada and Nagata 1994a,b), the adrenal gland (Ito and Nagata 1996, Liang et al., 1999), the brain (Cui et al., 1996) and the eye (Gunarso 1984, Gunarso et al., 1996, 1997, Kong and Nagata 1994, Cui et al., 2000) of mice, rats and chickens. Thus, it is clear that all the cells in various organs of various animals synthesize DNA and RNA not only in their nuclei but also in their mitochondria.

The relationship between the cell cycle and the intramitochondrial DNA as well as RNA syntheses was formerly studied in synchronized cells and it was clarified that both the intramitochondrial DNA and RNA syntheses were performed without any nuclear involvement (Nagata 1972a). However, the relationship between the aging of individual animals and the DNA and RNA syntheses has not yet been clarified except a few papers recently published by Korr and associates on mouse brain (Korr et al., 1997, 1998, Schmitz et al., 1999a,b). They reported both nuclear DNA repair, measured as nuclear unscheduled DNA synthesis, and cytoplasmic DNA synthesis labeled with \(^{3}H\)-thymidine in several types of cells in brain such as pyramidal cells, Purkinje cells, granular cells, glial cells, endothelial cells, ependymal cells, epithelial cells as observed by only light microscopic radioautography using paraffin sections. They observed silver grains over cytoplasm of these cells by light microscopy and maintained that it was reasonable to interpret these labeling as \(^{3}H\)-DNA outside the nuclei, which theoretically belonged to mitochondrial DNA without observing the mitochondria by electron microscopy. From the results, they concluded that distinct types of neuronal cells showed a decline of both unscheduled DNA and mitochondrial DNA syntheses with age in contrast with other cell types, glial and endothelial cells, which did not
show such age-related changes without counting the number of mitochondria in respective cells or counting the number of labeled mitochondria or calculating the labeling indices of mitochondria at respective aging stages. Thus, their results from the statistics obtained from the cytoplasmic grain counting seems to be not accurate without observing mitochondria directly by electron microscopy.

To the contrary, we first showed the relationship between the DNA synthesis and aging in hepatocytes of mice in vivo at various ages by means of electron microscopic radioautography observing the small dot-like silver grains, due to incorporations of $^3$H-thymidine, which were developed with phenidon developer after gold latensification exactly localized inside the mitochondria (Nagata and Ma 2005a). We demonstrated that increases and decreases were observed in the mitochondrial numbers and the numbers of labeled mitochondria as well as labeling indices of DNA synthesis with $^3$H-thymidine incorporations by direct observation on mitochondria at electron microscopic level.

Moreover, the radioautograms in the present study showing incorporations of $^3$H-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 many silver grains or not in respective cell types of pulmonary cells. With regards to RNA in mitochondria in animal cells or plastids in plant cells, many studies have been reported in various cells of various plants and animals since 1960s by biochemical approaches (Knight 1969, Chi and Suyama 1970) or by biochemical and morphological approaches (André and Marinozzi 1965, Swift 1965, O’Brien and Kalf 1967a,b, Schatz 1970, Tandler and Hoppel 1972). Most of these authors observed mitochondrial ribosomes appeared in the matrix compartment as small particles, 10-15 nm in diameter, by electron microscopic observation, which were extracted with RNase. The 3 classes of RNA, i. e., messenger, transfer and ribosomal, were also found in mitochondria (Schatz 1970). Mitochondria of various cells contained aminoacyl-RNA synthetase and DNA dependent RNA polymerase (Schatz 1970). The evidence for RNA synthesis in mitochondria was first demonstrated by means of electron microscopic radioautography incorporating $^3$H-uracil in lower organisms such as Agaricus campestris by Vogel and Kemper (1967) or $^3$H-uridine in Ochromonas by Gibbs (1968), then in animal cells such as chicken and mouse liver by André (1968) and Curgy (1968, 1970), in rat adrenocortical cells by Nussdorfer and Mazzochi (1971), in human cells such as HeLa cells by Nagata (1969, 1972a,b,c,d).

Most of these authors who reported intramitochondrial DNA and RNA synthese, however, used old-fashioned developers consisting of methol and hydroquinone (MQ-developer) which produced coarse spiral silver grains resulting in inaccurate localization over cell organelles, especially mitochondria, when observed by electron microscopy. Thus, most of these authors, except Nagata (1969, 1972a,b,c,d), showed photographs of electron radioautographs with large spiral-formed silver grains (2-3 μm in diameter) localizing not only over the mitochondria but also outside the mitochondria. In order to obtain smaller silver grains, we first used elon-ascorbic acid developer after gold latensification (Nagata et al., 1967a,b, Nagata 1969, 1972a,b,c,d), which produced comma-shaped smaller silver grains (0.3-0.8 μm in diameter), then later we used phenidon developer after gold latensification, producing dot-like small silver grains (0.2-0.3 μm in diameter) localizing only inside the mitochondria showing ultrahigh resolution of radioautograms (Murata et al., 1979, Nagata 1992, 1996, 1997, 2002). These papers (Nagata et al., 1967a,b, Nagata 1972a) were the first which demonstrated intramitochondrial RNA synthesis incorporating $^3$H-uridine with accurate intramitochondrial localization in avian and mammalian cells. With regards to the resolution of electron microscopic radioautography, on the other hand, many authors discussed the sizes of silver grains under various conditions and calculated various values of resolutions (Salpeter et al., 1969, Nadler 1971, Uchida and Mizuhira 1971, Nagata 1972c,d). Those authors who used the M-Q developers maintained the resolution to be 100-160 nm (Caro 1962, Salpeter et
al., 1969, Nadler 1971), while those authors who used the elon-ascorbic acid developer (Uchida and Mizuhira 1971, Nagata 1972c,d) calculated it to be 25-50 nm. When we used phenidon developer at 16°C for 1 min after gold latensification, we could produce very fine dot-shaped silver grains and obtained the resolution around 25 nm (Murata et al., 1979, Nagata 1992, 1996, 1997). For the analysis of electron radioautographs, Salpeter et al., (1969) proposed to use the half-distance value (HD) and very complicated calculations through which respective coarse spiral-shaped silver grains were judged to be attributable to the radioactive source in a certain territory within a resolution boundary circle. However, since we used phenidon developer after gold latensification to produce very fine dot-shaped silver grains, we judged only the silver grains which were located in the mitochondria which were dot-shaped very fine ones to be attributable to the mitochondria without any problem as was formerly discussed (Nagata 1972c,d). Blacket and Perry (1977) later proposed to use the simplified method of hypothetical grain analysis under the same conditions. However, since we used Konica NR-H2 radioautographic emulsion which had smaller sized silver bromide crystals than the above mentioned Kodak or Ilford and we used phenidon developer after gold latensification to produce very fine dot-shaped silver grains instead of D-19, thus resulting in smaller sized dot-shaped silver grains as small as 50 nm in diameter with a smaller HD as small as 25 nm, we judged only the silver grains localizing over the mitochondria to be attributable to the mitochondria without any problem as was formerly discussed (Nagata 1972c,d). As for the section thickness, we used thicker semithin sections at 0.2μm thickness which did not effect the HD value of this experiment since we used tritium as RI which emitted beta rays with very low energy having very short range as 0.2μm in the emulsions. Thus, the number of labeled mitochondria as well as labeling index which were calculated from the numbers of mitochondria over which the silver grains really existed without adding any hypothetical silver grains which should be less than 10% (only several%) if added under the experimental conditions that we carried out in this experiment.

Anyway, we first showed the relationship between the DNA and RNA syntheses and aging in liver cells of mice in vivo at various ages by means of electron microscopic radioautography observing the small dot-like silver grains, due to incorporations of $^3$H-thymidine and $^3$H-thymidine, which exactly localized inside the mitochondria (Nagata and Ma 2005a,b). We demonstrated that increases and decreases were observed in the mitochondrial numbers and labeling indices of DNA synthesis with $^3$H-thymidine incorporations (Nagata and Ma 2005a) or RNA synthesis with $^3$H-uridine incorporations (Nagata and Ma 2005b) by direct observation on mitochondria at electron microscopic level. Thus, this paper should be the first to show the relationship between the DNA and RNA synthesis and aging in pulmonary cells of mice in vivo at various ages by means of electron microscopic radioautography observing the small dot-like silver grains, due to incorporations of $^3$H-thymidine and $^3$H-uridine, which exactly localized inside the mitochondria. We formerly studied the relationship between the RNA synthesis and aging in the adrenal glands of mice at various ages from postnatal day 4 to month 6 and  and found that the numbers of total mitochondria and the numbers of labeled mitochondria with incorporations of $^3$H-uridine in respective adrenal cortical zones, zona glomerulosa, fasciculata, reticularis and the adrenal medulla cells increased from postnatal day 14 to month 12 (Liang et al., 1999). However, the animals at the perinatal stages from prenatal day to postnatal day 14, as well as the senescent stage at postnatal year 2, were not yet studied. There was a discrepancy between the results obtained from the adrenal glands which showed only increases of the total mitochondria and the labeled mitochondria with aging and the present results from the pulmonary cells which showed increases and decreases with aging. The reason for this difference might be the difference between the cell types (adrenal cells and pulmonary cells).

From the results obtained in the present study, it was concluded that almost all the pulmonary cells of mice at various ages, from prenatal embryos to postnatal newborn, young,
adult and senescent animals, were labeled with silver grains showing DNA and RNA synthesis with $^3$H-thymidine and $^3$H-uridine in their mitochondria. The results obtained from the lung at present should form a part of special radioautographology (Nagata 2002), i.e., application of radioautography to the lung, as well as a part of special cytochemistry (Nagata 2001). We expect that such special radioautographology and special cytochemistry should be further developed in all the organs in the future.

CONCLUSIONS

On electron microscopic radioautograms obtained from 11 groups of aging mice labeled with either $^3$H-thymidine or $^3$H-uridine, the number of mitochondria, the number of labeled mitochondria and the mitochondrial labeling index labeled with either $^3$H-thymidine or $^3$H-uridine showing DNA or RNA syntheses in each pulmonary cells, type I and II epithelial cells, interstitial cells and endothelial cells, were counted and the averages in respective aging groups were compared.

The present results demonstrated that the numbers of mitochondria, the numbers of labeled mitochondria and the labeling indices of intramitochondrial DNA and RNA syntheses in respective cells of mice at various ages increased and decreased according to aging of animals.

From the present results it was concluded that both mitochondrial DNA and RNA syntheses in 4 types of pulmonary cells showed aging changes.

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