Int J Biol Med Res. 2011; 2(3): 702-708

Contents lists available at BioMedSciDirect Publications

International Journal of Biological & Medical Research

Journal homepage: www.biomedscidirect.com

Original Article

Radioautographic studies on DNA synthesis of the lungs of aging salamanders

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ARTICLE INFO

Keywords:
DNA synthesis
Light microscopic radioautography
Salamanders
Pulmonary cells
Aging

ABSTRACT

The DNA synthesis and morphological changes of the lungs of the salamanders, *Hynobius nebulosus,* were studied by light microscopic radioautography. The lung tissues of 21 salamanders in 7 groups at various aging stages, from juvenile animals at 4, 6 and 8 weeks after metamorphosis, young adults at 3, 8 and 12 months after metamorphosis, and senescent adults at 5 years after metamorphosis were used for this study. They were injected intraperitoneally with 3H-thymidine (370 KBq/g body weight), and after 1 hr the lung tissues were fixed in buffered 2.5% glutaraldehyde solution for 2 hr and postfixed in 1% osmium tetroxide solution for 1 hr. The tissues were embedded in epoxy resin Quetol-812. Thick sections were cut at 2 μm on a Porter-Blum MT-6000 ultramicrotome and were coated with Konica NR-M2 emulsion by a dipping procedure for light microscopic radioautography. After exposure for 2 months, the radioautographs were developed in SDX-1 developer, stained with 0.1% toluidine blue and were observed with an Olympus Vanox light microscope, and analyzed quantitatively. As the results, the labeling indices in the nuclei of the pneumocytes, the mucous cells, the basal cells and the ciliated cells in the superficial layer as well as the fibroblasts and the endothelial cells in the deep layer changed due to aging. The labeling indices of the 3 types of cells in the superficial layer, the mucous cells, the pneumocytes and the basal cells were high from 4 weeks to 8 weeks but dropped down at 8, 12 and 60 months. These results showed that the 3 types of cells proliferated in the early stages of development from 4 to 8 weeks and completed the development in the adult stages. The labeling index of the ciliated cells was very low, which showed that they had no activity to proliferate but replaced by the immature basal cells which differentiated to the ciliated cells. To the contrary, the labeling indices of the 2 cell types in the deep layer, the fibroblasts and the endothelial cells, were lower than the cells in the superficial layer and kept very low levels at 8, 12 and 60 months after metamorphosis. Thus, it was concluded that the 4 types of cells in the superficial layer belonged to the renewing cell population and the 2 types of cells in the deep layer to the stable cell population.

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1. Introduction

The technique of light and electron microscopic radioautography is an excellent tool to demonstrate the localization and intensity of the macromolecular synthesis such as nucleic acids, proteins, glucides and lipids during the development of various tissues and organs in various kinds of animals including man (Nagata, 1995, 1997, 1998b, 2002). Studies on the aging changes of the DNA synthesis of various organs in some experimental animals such as mice and rats have been carried out in our laboratory since many years (Chen et al., 1995; Duan et al., 1993; Gao, 1993; Gao et al., 1992, 1993a, 1993b, 1995a, 1995b; Hanai, 1993; Hanai and Nagata, 1994a, 1994b; Hayashi et al., 1993; Ito, 1996; Ito and Nagata, 1996; Jin, 1996; Jin and Nagata, 1995a, 1995b; Kong, 1993; Kong and Nagata, 1995; Kong et al.,

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1992; Li, 1994; Li and Nagata, 1995; Ma, 1988; Ma and Nagata, 1988, 1990; Morita et al., 1994; Nagata, 1992, 1994, 1995, 2002; Nagata and Ma, 2003; Nagata and Usuda, 1986; Olea and Nagata, 1992; Yamada and Nagata, 1992). With regards to the radioautographic investigation on the incorporation of 3H-thymidine into the tissues in the salamanders no literature has been found out except a paper reported on the bone and the skin of this animal (Nagata, 1998c). Moreover, the systematic studies on age related changes of the DNA synthesis in the pulmonary cells in the salamanders at various aging stages from metamorphosis to adult and senescent stages have not yet been performed. The salamander, Hynobius nebulosus, is one of the amphibious animals which is commonly found in small rivers in the high mountains in Japan such as the Japan Alps at the central part of Honshu in Japan. The animal possesses the lung as the respiratory organ after metamorphosis similar to the lungs of the reptile, ornithic and mammalian animals. It should be very interesting to study the macromolecular synthesis of the lung tissues of aging salamanders and compare the results with the mammalian tissues from the point of view of comparative anatomy and histology. In order to obtain the information on the DNA synthesis of pulmonary cells in the respective individuals in the aging salamanders, we investigated the age related changes of DNA synthesis and morphological changes in the lungs after metamorphosis from day 28 (4 weeks) to 60 months (5 years) by light microscopic radioautography using 3H-thymidine incorporations.

2. Materials and Methods

2.1. Animals

Twenty-one salamanders in 7 groups at various aging stages, each consisting of 3 individuals of both sexes, aged from juvenile animals at 4, 6 and 8 weeks after metamorphosis, young adults at 3, 8 and 12 months after metamorphosis, and senescent adults at 5 years (60 months) after metamorphosis were used for this study. They were bred in our laboratory and were housed under conventional conditions, fed with normal diet with access to water ad libitum. As for the materials used in our studies cited here, all the procedures relating to the human materials and animal experiments were in accordance with the protocol reviewed and approved by either the ethical standards laid down in the 1964 Declaration of Helsinki revised 2000 and the guidelines of the principles of laboratory animal care (NIH publication No. 85-23, revised 1985) as well as either the Ethics Committee of Shinshu University School of Medicine and the Animal Research Committee of Shinshu University School of Medicine.

2.2. Radioautography

All the animals were injected intraperitoneally with 3H-thymidine (NEN, New England Nuclear, Boston, MA, USA, specific activity 185 MBq/mM) at a concentration of 370 KBq/g body weight and they were sacrificed after 1 hour by decapitation. The lung tissues from the right upper lobes were taken out, cut into small tissue pieces and fixed by immersion fixation in cold 2.5% glutaraldehyde solution in 0.1 M cacodylate buffer (pH 7.4) at 4˚C for 2 hr, and postfixed in cold 1% osmium tetroxide in the same buffer at 4˚C for 1 hr. The tissue blocks were rinsed several times in 0.1 M cacodylate buffer (pH 7.4), dehydrated in a graded series of ethanol and acetone, embedded in an epoxy resin Quetol-812(Oken, Tokyo, Japan). After polymerization, thick sections Japan). were cut at 2 μm thickness on a Porter-Blum MT-6000 ultramicrotome (Dupont-Sorvall, Newtown, MA, USA), and coated with Konica NR-M2 light microscopic radioautographic emulsion (Konica Co., Tokyo, Japan) by a dipping method (Nagata, 1992; 1998a, 2002). The coated slides were placed in light-proof slide boxes and exposed in a refrigerator at 4˚C for 2 months, developed in SDX-1 developer (Konica, Tokyo, Japan) at 20˚C for 5 min. After development, the slides were fixed in acid fixer for 10 min., rinsed 3 times in distilled water and stained with 0.1% toluidine blue solution. The radioautographs were observed and photographed with an Olympus Vanox AHB-LB light microscope (Olympus, Tokyo,

2.3. Quantitative Analysis

In order to calculate the labeling indices of pulmonary cells in the lung tissues, three specimens from each animal were used for analysis. From each slide 100 cells and altogether 300 cells in the lung of each animal, respectively, were counted randomly under an oil immersion objective and the number of labeled cells with 3H-thymidine was recorded. A cell was considered labeled if 5 or more silver grains were observed over the nucleus of the cell. The labeling index of each cell type in the superficial and the deep layers at each time interval was calculated by counting total 900 cells, 300 each, of 3 individuals in each age group. The labeling indices were calculated with a personal computer (Apple Macintosh LC475, Tokyo, Japan) and were expressed as mean ± S.D. (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 $P \leq 0.05$.

3.Results

3.1.Qualitative Observation

The lung tissues of salamanders are composed of two layers, the superficial layer and the deep layer. The former consists of four kinds of pulmonary cells, the pneumocytes, the mucous cells, the ciliated cells and the basal cells, while the latter consists of two kinds of pulmonary cells, the interstitial cells (fibroblasts) and the endothelial cells (Figs. 1-5).

Observing the light microscopic radioautograms of the lungs of salamanders at various aging stages, many nuclei of respective pulmonary cells are labeled with silver grains due to incorporations of 3H-thymidine, demonstrating the DNA synthesis (Figs. 1-4). Light microscopic radioautograms of the lungs of juvenile salamanders at 4 and 6 weeks after metamorphosis showed that the pulmonary cells at this stage consisted of 2 immature layers, the superficial layer and the deep layer (Fig. 1). The superficial layer consisted of the pneumocytes, the mucous cells, the basal cells and very few of the ciliated cells which appeared cuboidal in shape, while the deep layer consisted of the interstitial cell or elongated fibroblasts surrounded with collagen fibers and the endothelial cells which formed the blood capillaries. Among these cells in the radioautograms only a few pneumocytes and mucous cells are labeled. At 8 weeks after metamorphosis, the light microscopic radioautograms of the lungs of the salamanders at this stage showed much more labeling cells than the previous stages at 4 and 6 weeks after metamorphosis. The superficial layer at this juvenile stage consisted of 4 types of cells, the pneumocytes, the mucous cells, the ciliated cells and the basal cells which

appeared cuboidal in shape, but many nuclei of the pneumocytes and the mucous cells are labeled with 3H-thymidine (Fig. 2). The labeled nuclei are observed in all cell types in the superficial layer, the pneumocytes, the mucous cells, the basal cells and very few ciliated cells. The labeled nuclei were also observed in the interstitial cells and the endothelial cells in the deep layer (Fig. 3). The light microscopic radioautograms of the lungs of young adult salamanders at 3 (Fig. 4) and 8 months after metamorphosis showed the morphology of mature pulmonary cells. The pneumocytes, the mucous cells in the superficial layer changed their shapes to flattenedsquamous, while the other ciliated cells and the basal cells remained cuboidal. Some of the flattened cells, the pneumocytes and the mucous cells are labeled (Fig. 4), but the number of labeled cells decreased as compared to the previous stage. Almost no cells in the ciliated cells and basal cells were labeled at this stage. The light microscopic radioautograms of the lungs of aged adult salamanders at 12 months after metamorphosis showed both the superficial and deep layers flattened (Fig. 5). Only a few cells in respective cell types were found and the number of labeled cells decreased as compared with the previous stages. Finally, the light microscopic radioautograms of the lungs of senescent salamanders at 5 years after metamorphosis, all the cells in both the superficial layer and the deep layer remained flattened and no labeled cell was found in respective cell types at this stage.

Figs. 1-5. Light microscopic radioautograms of the lungs of salamanders at various aging stages. Several pulmonary cells are labeled with silver grains due to DNA synthesis incorporating 3H-thymidine. Magnification. x 400.

Fig. 1. Light microscopic radioautogram of the lung of a salamander at 4 weeks after metamorphosis. The pulmonary cells at this stage consist of immature superficial layer at the bottom right and the deep layer at the top left. The superficial layer consists of the pneumocytes and the mucous cells, while the deep layer consists of the interstitial cell (or fibroblasts) and the endothelial cells forming the capillaries. The pneumocytes and the mucous cells in this juvenile stage appearcuboidal in shape. Only a few pneumocytes and mucous cellsare labeled.

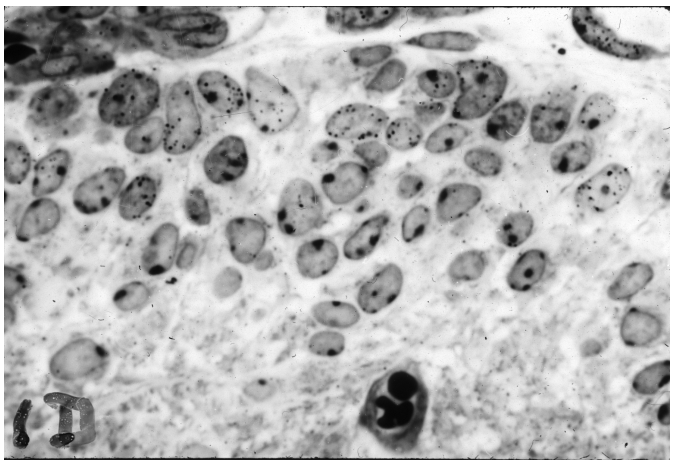


Fig. 2. Light microscopic radioautogram of the lung of a salamander at 6 weeks aftermetamorphosis. The pneumocytes and the mucous cells in this juvenile stage appearcuboidal in shape. Many pneumocytes and mucous cells are labeled with 3H-thymidine at this stage.

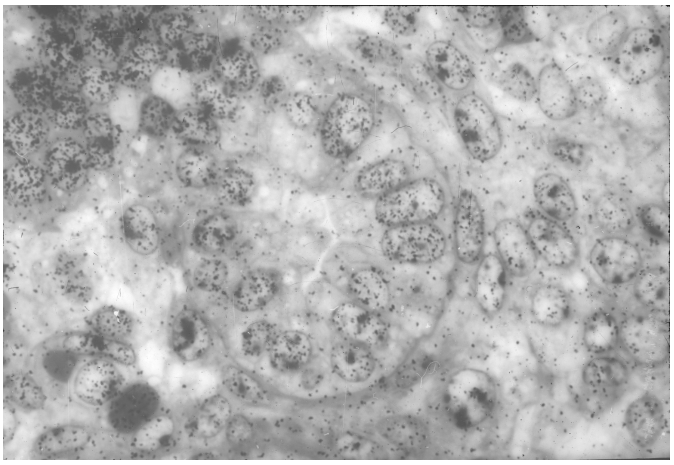


Fig. 3. Light microscopic radioautogram of the lung of a salamander at 8 weeks after metamorphosis. The pneumocytes, the mucous cells and the basal cells in the superficial cells appear cuboidal. Some of the cuboidal cells possess several cilia, appearing as the ciliated cells. The labeled cells are observed in both the superficial layer and the deep layer except the ciliated cells.

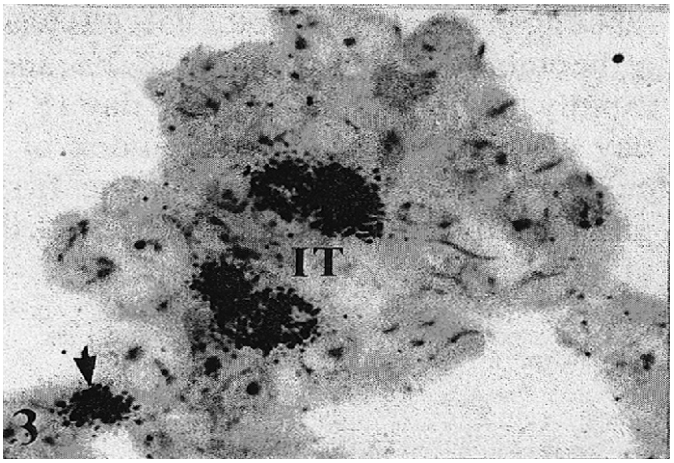


Fig. 4. Light microscopic radioautogram of the lung of an adult salamander at 8 months after metamorphosis. The pneumocytes and the mucous cells in the superficial layer changed their shapes to flattenedsquamous, while the ciliated cells remained cuboidal. However, the deep layer cells, the fibroblasts and the endothelial cells appear flattened. Some of the flattened cells, fibroblasts, are labeled, but the number of labeled cells decreased as compared to the previous stage.

Fig. 5. Light microscopic radioautogram of the lung of an adult salamander at 12 months after metamorphosis. The pneumocytes and the mucous cells in the superficial layer as well as the fibroblasts and the endothelial cells changed their shape to flattenedsquamous. No labeled cell is found at this stage.

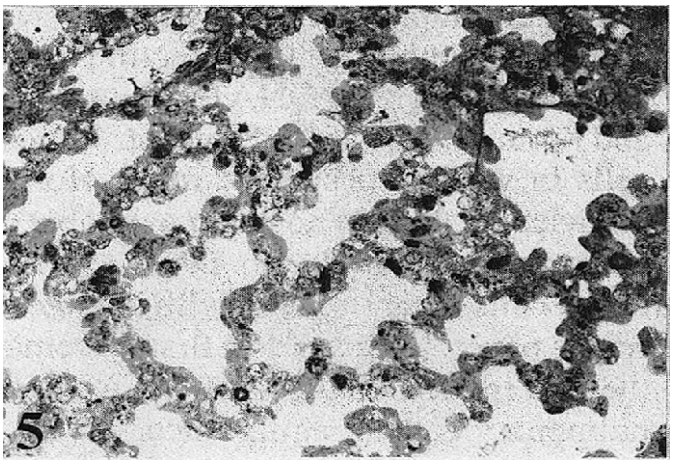
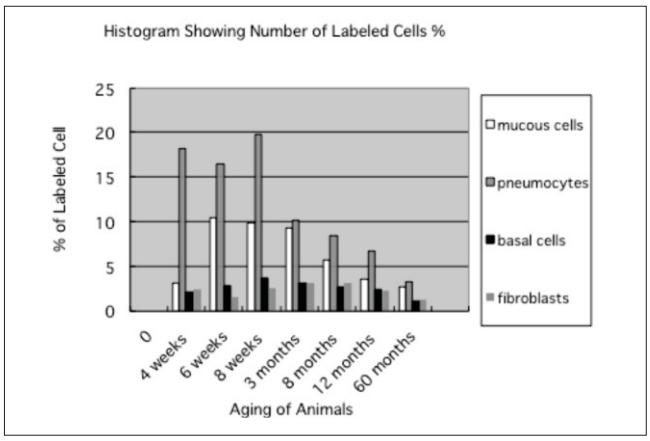


Fig. 6. Histogram showing the labeling indices of the pulmonary cells in the lungs of salamanders at various aging stages from 4 weeks to 5 years after metamorphosis. Mean + S.D.



3.2.Quantitative Observation

The labeling indices of respective cell types changed with aging as expressed by mean in each aging group (Fig. 6). In general, the labeling indices of the deep layer cells were lower than the superficial layer cells. The labeling indices of the mucous cells rapidly increased from 4 weeks to 6 weeks after metamorphosis about 3-5 %, then increased to about 10 % at 8 and 12 months and finally decreased to 2-3% at 60 months. The labeling indices of the pneumocytes decreased from 18% at 4 weeks to 16% at 6 weeks, then increased to 20% at 8 weeks and then gradually decreased to 10% at 3 months and fell down to 3% at 60 months. The labeled cells in the ciliated cells and basal cells in the superficial layer were very few at 4 and 6 weeks, but the labeled basal cells increased from 6 weeks to 8 weeks. The labeling indices of the basal cells reached the peak about 9% at 8 weeks, then decreased and kept very low level around 0-1% from 3 months to 60 months after metamorphosis. On the other hand, the labeling indices of both the fibroblasts and the endothelial cells in the deep layer showed very low levels about 2-5% during the aging from 4 weeks to 60 months without showing any increase and decrease.

4. Discussion

Radioautography of various cells and tissues labeled with 3H-thymidine provides a useful tool for the detection of nuclei of cells which are synthesizing DNA (Nagata, 1995, 1997, 1998b, 2002). Schultze and Oehlert (1960) and Edwards and Klein (1961) formerly studied the distribution of S-phase cells in various tissues of mice and rats. We have studied the aging changes of the DNA synthesis of various organs in mice (Chen et al., 1995; Duan et al., 1993; Gao, 1993; Gao et al., 1992, 1993a, 1993b, 1995a, 1995b ;Hanai, 1993; Hanai and Nagata, 1994a, 1994b; Hayashi et al., 1993; Ito, 1996; Ito and Nagata, 1996; Jin, 1996; Jin and Nagata, 1995a, 1995b; Kong, 1993; Kong and Nagata, 1995; Kong et al., 1992; Li, 1994; Li and Nagata, 1995; Ma, 1988; Ma and Nagata, 1998, 1990; Morita et al., 1994; Nagata, 1994, 1995, 1997, 1999, 2002, 2004, 2005, 2006, 2007, 2008, 2009, 2010; Nagata and Ma, 2003; Nagata and Usuda, 1986; Nagata et al., 2000a, 2000b; Olea and Nagata, 1992; Sun et al., 1994, 1995, 1997; Yamada and Nagata, 1992). However, there is no paper available dealing with the systematic study on the DNA synthesis of various cells and tissues in salamanders except a paper published by the present authors several years ago (Nagata, 1998c).

In this paper, we first provide a systematic study on the age related changes of the DNA synthesis in respective cell types of the lungs of aging salamanders from 4 weeks to 60 months after metamorphosis. We demonstrated that the results obtained by radioautography showed the activity of DNA synthesis of all the cell types, the pneumocytes, the mucous cells, the ciliated cells, the basal cells in the superficial layer and the fibroblasts and the endothelial cells in the deep layer of the lungs of the aging salamanders. However the transitions of labeling indices of respective cell types were quite different.

The labeling indices of the mucous cells in the superficial layer of the lungs increased from 4 weeks after metamorphosis to 6 weeks and reached the peak at 6 weeks after metamorphosis, then showed a gradual reduction from 8 weeks to 12 months and finally fell down to 60 months onwards. The labeling indices of the pneumocytes in the superficial layer showed a little different transition. It showed a peak at 4 weeks after metamorphosis and slightly decreased at 6 weeks, then slightly increased at 8 weeks, reaching a peak, then fell down gradually to 60 months. On the other hand, the labeled nuclei in the ciliated cells and the basal cells were very few at 4 and 6 weeks, but they increased at 8 weeks, then decreased again at 3 months. The labeling indices of the basal cells showed a small peak at 8 weeks and kept very low level from 3 months to 60 months, while the labeling indices of the ciliated cells kept very low level from 4 weeks to 60 months after metamorphosis. These results showed that the 3 types of cells, the mucous cells, the pneumocytes and the basal cells in the superficial.

and 8 weeks, in contrast that the ciliated cells did not show any DNA synthetic activity and proliferative activity. Thus, it is suggested that the pneumocytes, the mucous cells and the basal cells could proliferate by themselves, but the ciliated cells could not and they might be substituted by the differentiation of the basal cells. However, the labeling indices of the fibroblasts and the endothelial cells in the deep layer were quite different. The labeling indices of both the fibroblasts and the endothelial cells kept low levels around a few percents from 4 weeks to 60 months after metamorphosis and did not show any peak. These results mean that both the fibroblasts and the endothelial cells in the deep layer did not show so much activity to synthesize DNA and no rapid proliferations during the development from 4 weeks to 60 months. These results were in agreement with the studies made in other organs in other animals such as the skeletal muscle of mouse (Hayashi et al., 1993), the spleen of mouse (Olea and Nagata, 1992), the salivary gland of mouse (Chen et al., 1995, Nagata et al. 2000a), the esophagus of mouse (Duan et al., 1993), the small intestines of mouse (Morita et al., 1994), the colon of mouse (Jin, 1995), the caecum of mouse (Jin and Nagata, 1995a, 1995b), the liver of mouse (Ma, 1988; Ma and Nagata, 1988, 1990; Nagata and Ma, 2003, Nagata 2007, 2008, 2009, 2010), the pancreas of mouse (Nagata, 1994, 1995, 1997; Nagata and Usuda, 1986), the trachea of mouse (Sun et al., 1997), the lung of mouse (Kauffman, 1975; Sun et al., 1994, 1995, 1997), the lung of rat (O'Hare and Townes, 1970; Kauffman et al. 1974), the kidney of mouse (Hanai, 1993; Hanai and Nagata, 1994, 1994b, Nagata 2005); the testis of mouse (Gao,1993; Gao et al., 1995, 1993b, 1995a, 1995b), the ovary and uterus of mouse (Li, 1994; Li and Nagata, 1995), the decidual cells of mouse (Yamada and Nagata, 1992), the adrenal gland of mouse (Ito, 1996; Ito and Nagata, 1996, Nagata 2009), the retina of mouse (Gao et al., 1992, 1993b; Kong, 1993; Kong and Nagata, 1995; Kong et al., 1992), the cornea of mouse (Gao et al. 1993a), showing that some cell types could proliferate by themselves by synthesizing DNA while some other cell types could not proliferate at aged stages and might be substituted by other cell types.

With regards to the DNA synthesis and proliferation of various cell types in the salamanders, however, only the data concerning the cartilage and the epidermis was studied (Nagata 1998, 2007), but no literature was published concerning the DNA synthesis in the lungs of the salamanders. The spurt of DNA synthesis of 3 types of cells in the superficial layer, the mucous cells, the pneumocytes and the basal cells, of the aging salamanders immediately after metamorphosis, as observed from 4 weeks to 8 weeks, may reflect the immaturity of the cells in these tissues. However, it dropped down as the growth proceeded at 8 and 12 months and finally decreased to a lower level, but never dropped down at 8, 12 and 60 months after metamorphosis. These results show that the 3 types of cells proliferated in the early stages of development from 4 to 8 weeks and completed the development in the adult stage at 8 months after metamorphosis and then kept a lower DNA synthetic activity. To the contrary, it is worthy of notice that the DNA synthetic activity of the ciliated cells was very low, which showed that such mature cell types as the ciliated cells had no activity to proliferate by synthesizing DNA but replaced by the immature basal cells which differentiated to the ciliated cells. On the other hand, the 2 cell types in the deep layer, the fibroblasts and the endothelial cells, were lower than the cells in the superficial layer and kept very low levels of DNA synthetic activity at 8, 12 and 60 months after metamorphosis.The difference between these two cell populations should be due to the difference that the 4 types of cells in the superficial layer, the mucous cells, the pneumocytes and the basal cells including the ciliated cells, belong to the renewing cell population, while the other 2 types of cells in the deep layer, the fibroblasts and the endothelial cells, belong to the stable cell population according to the theory postulated byLeblond (1965, 1981) in his review on 3H-thymidine radioautography.

In this paper we investigated the age related changes of the DNA synthesis of several types of cells in the lungs of aging salamanders from juvenile stages at 4, 6 and 8 weeks after metamorphosis, young adult stages at 3, 8 and 12 months after metamorphosis, and finally adult senescent stage at 5 years after metamorphosis, which resulted in very interesting findings. The results constitute supplemental studies on the special radioautographology or the radioautographology of the organs as was proposed by Nagata (1998b, 2002) as well as on the special cytochemistry (Nagata, 2001). The age related changes of the RNA synthesis of these cells should be also very interesting, which has not yet been performed. It requires further investigation on this problem in the future.

5. Conclusions

1) The activity of DNA synthesis of the pneumocytes, the mucous cells, the basal cells and the ciliated cells in the superficial layer and the fibroblasts and the endothelial cells in the deep layer of the lungs of salamanders at various aging stages changed due to aging.
2) The labeling indices of the 3 types of cells in the superficial layer, the mucous cells, the pneumocytes and the basal cells were high from 4 weeks to 8 weeks but dropped down at 8, 12 and 60 months. These results show that the 3 types of cells proliferated in the early stages of development from 4 to 8 weeks and completed the development in the adult stages.
3) To the contrary, the DNA synthetic activity of the ciliated cells was very low, which showed that they had no activity to proliferate by synthesizing DNA but replaced by the immature basal cells which differentiated to the ciliated cells.
4) The labeling indices of the 2 cell types in the deep layer, the fibroblasts and the endothelial cells, were lower than the cells in the superficial layer and kept very low levels at 8, 12 and 60 months after metamorphosis.
5) These results demonstrate that the 4 types of cells in the superficial layer belong to the renewing cell population and the 2 types of cells in the deep layer to the stable cell population.

Acknowledgments

This study was supported in part by a Grant-in-Aids for Scientific Research from the Ministry of Education, Science and Culture of Japan (No. 501010, 501533, 56870001, 58015046, 02454564) as well as a Grant for Promotion of Characteristic Research and Education from the Japan Foundation for Promotion of Private Schools (1997, 1998, 1999, 2000) given to the author.

The author is grateful to Dr. Kiyokazu Kametani, Technical Official, Research Center for Instrumental Analysis, Shinshu University, for his technical assistance during the course of this study.

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