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Cell Senescence as Observed by Electron Microscopic Radioautography

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1. Introduction

The term "cell senescence" initially means how the cells change when they get old due to their aging. It contains 2 meanings, one how a cell changes when it is isolated from in vivo original animals or plants such as in vitro cells in cell culture, while the other means how all the cells of an animal or a plant change in vivo due to the aging of the individual animal or plant. In order to study the cell senescence, we have 2 research techniques to clarify how cells get old, i.e., morphological technique and functional technique. The former employs microscopy either light microscope or electron microscope to observe the structure of cells and tissues, while the latter employs functional techniques such as physiological or biochemical to observe either the electric activities or chemical components. Since I am anatomist and had learned morphological technique, I employed to observe cells by light and electron microscopy. I had first studied the meaning of cell senescence many years ago (more than 50 years) how a cell changed when it was isolated from original experimental animals such as mice and rats by cell culture (Nagata 1956, 1957a,b), and then moved to the study on the latter cell senescence, i.e., how all the cells of an experimental animal change in vivo due to the senescence of the individual animal bodies (Nagata 1959, 1962, Nagata and Momoze 1959, Nagata et al. 1960a,b).

Recently, I have been studying the senescent changes from the viewpoint of the cell nutrients which were incorporated and synthesized into various cells in individual animals during their senescence (Nagata 2010c). Therefore, this article deals with the cell senescence of animal cells in vivo, how the metabolism, i.e., incorporations and syntheses of respective nutrients, the macromolecular precursors, in various kinds of cells change due to the senescence of individual experimental animals such as mice and rats by means of microscopic radioautography. The incorporations and syntheses of various nutrients such as DNA, RNA, proteins, glucides, lipids and others in various kinds of cells of various organ in respective organ systems such as skeletal, muscular, circulatory, digestive, respiratory, urinary, reproductive, endocrine, nervous and sensory systems should be reviewed referring many original papers already published from our laboratory.

When I was first asked early this year (April 2011) from the publisher, named InTech, Open Access Publisher in Croatia, to contribute this article as well as to edit the articles submitted

from around 40 authors throughout the world, I initially intended to write only one chapter entitled "Senescence as Analyzed by Microscopic Radioautography." However, when I was almost finishing this article in one chapter which consisted of the text around 140 pages and around 30 figures which might become over 170 pages altogether, the publisher requested me to submit only one chapter within exactly 16, 18, 20, 22, 24 or 26 pages including both text and figures. Since I could not agree with them in submitting such a short chapter dealing with all the research results of myself and my associates, I insisted to submit the large article in one chapter including so much information as the results of my research on senescence for more 50 years since 1955 up to the present time 2011, they requested me to divide the original one chapter into 3 or 4 short chapters.

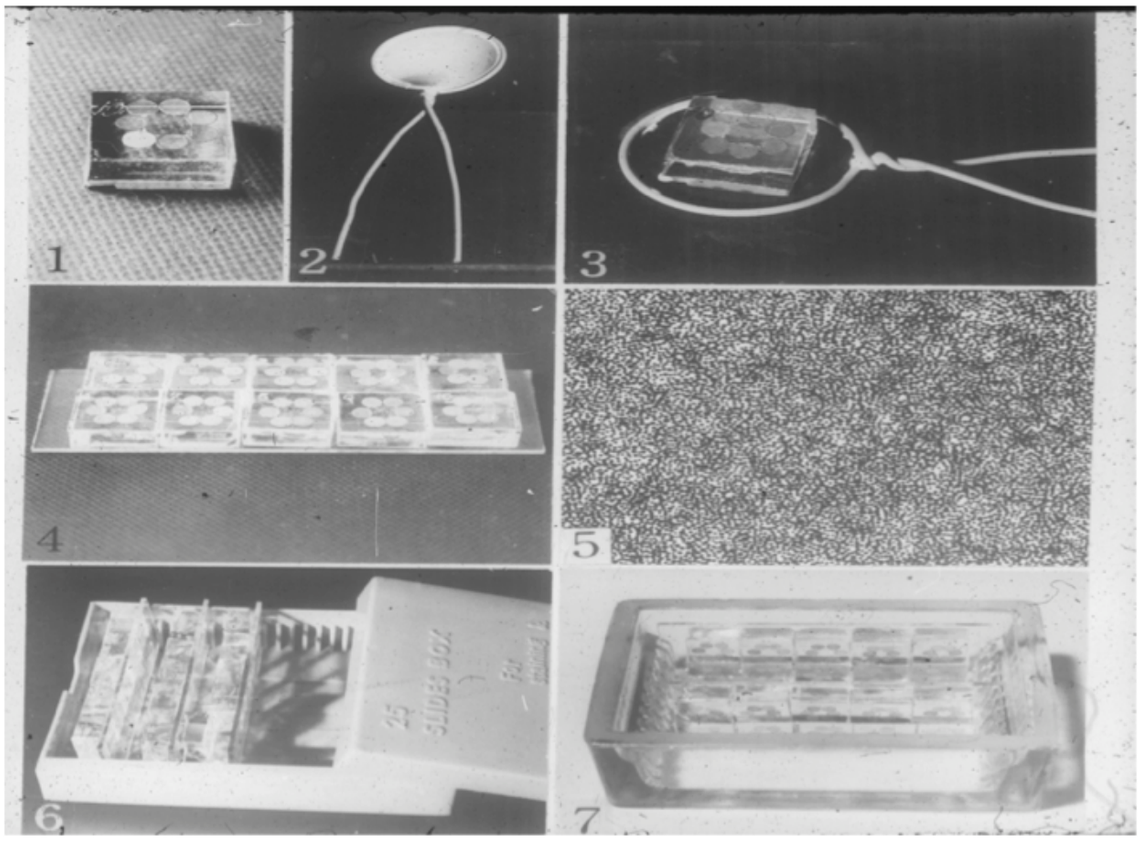
Thus, I tried to divide the original draft into 4 chapters, including the foundations of radioautography and the results of its application to all the organ systems, i.e., skeletal, muscular, circulatory, digestive, respiratory, urinary, reproductive, endocrine, nervous and sensory organs altogether. As the results of dividing the initial one chapter into 4, the final chapters were consisted of more than 26 pages as the publisher requested. However, I am going to submit the longer chapters to the publisher and insist to publish those chapters as they are, otherwise, I would rather prefer to withdraw them from this book and would like to contribute them to any other suitable publishers in the world.

This first chapter deals with the methodology of microscopic radioautography as well as the first parts of the applications of radioautography to the organ systems, i.e. the organ of movement (skeletal and muscular system) and the circulatory system.

1.1 Method in microscopic radioautography

For the purpose of observing the localizations of the incorporations and syntheses of various nutrients synthesizing macromolecules in the human and animal bodies such as DNA, RNA, proteins, glucides and lipids in various kinds of cells of various organ in respective organ systems such as skeletal, muscular, circulatory, digestive, respiratory, urinary, reproductive, endocrine, nervous and sensory systems, we employed the specific techniques developed in our laboratory during these 50 years (Nagata 2002). The technique is designated as radioautography using RI-labeled compounds. Some scientists use another term autoradiography which is used as the synonym to radioautogarphy. However, the author prefers the term radioautography because of the etymological reason (Nagata 1996b). To demonstrate the localizations of macromolecular synthesis by using such RI-labeled precursors as ^{3}H-thymidine for DNA, ^{3}H-uridine for RNA, ^{3}H-leucine for proteins, ^{3}H-glucosamine or $^{35}SO_4$ for glucides and ^{3}H-glycerol for lipids are divided into macroscopic radioautography and microscopic radioautography. The techniques employ both the physical techniques using RI-labeled compounds and the histochemical techniques treating tissue sections by coating sections containing RI-labeled precursors with photographic emulsions and processing for exposure and development. Such techniques can demonstrate both the soluble compounds diffusible in the cells and tissues and the insoluble compounds bound to the macromolecules (Nagata 1972b). As the results, specimens prepared for EM RAG (electron microscopic radioautography) are very thick than conventional EM specimens and should be observed with high voltage electron microscopes in order to obtain better transmittance and resolution (Nagata 2001a,b). Such radioautographic techniques in details should be referred to other literature (Nagata 2002). On the other hand,

the systematic results obtained by radioautography should be designated as radioauographology which means the science of radioautography (Nagata 1998b, 1999e, 2000e). This article deals with the results dealing with the radioautographic changes of individual cell by aging that should be included in radioautographology.



Explanation of Figures. From Nagata, T.: Acta Microsc. Vol. 6: Suppl. B. p. 42, 1997a. Brazil. Soc. Electron Microsc., San Paulo, Brazil

Fig. 1. Photographs showing the standard procedure for preparing EMRAG (electron microscopic radioautograms) by the wire-loop method (Nagata 1982, 1985).
Fig. 1-1. Six grid meshes carrying sections are placed on a square glass block.
Fig. 1-2. A large wire-loop is dipped into the melted radioautographic emulsion and a thin film of the emulsion is obtained.
Fig. 1-3. The emulsion film with the wire-loop (Fig. 1-2) is applied horizontally to the glass block on which 6 grid meshes were placed (Fig. 1-1).
Fig. 1-4. Ten glass blocks carrying 6 grid meshes each (Fig. 1-3) are attached on a glass slide with Scotch tape.
Fig. 1-5. The emulsion film picked up at random is checked by transmission electron microscopy before exposure. Note the monolayer arrangement of the silver bromide crystals in Konica NR-H2 emulsion in this figure. x6,000.
Fig. 1-6. Several glass slides, each carrying 10 glass blocks with grid meshes, are stored in a light tight slide box kept in a refrigerator at 4˚C for exposure.
Fig. 1-7. All the grid meshes on glass blocks are developed, fixed and stained simultaneously.

1.2 Macromolecular synthesis

The human body as well as the bodies of any experimental animals such as mice and rats consist of various macromolecules. They are classified into nucleic acids (both DNA and RNA), proteins, glucides and lipids, according to their chemical structures. These macromolecules can be demonstrated by specific histochemical staining for respective molecules such as Feulgen reaction (Feulgen and Rossenbeck 1924) which stains all the DNA contained in the cells. Each compounds of macromolecules such as DNA, RNA, proteins, glucides, lipids can be demonstrated by respective specific histochemical stainings (Pearse 1991) and such reactions can be quantified by microscpectrophotometry using specific wave-lengths demonstrating the total amount of respective compounds (Nagata 1972a). To the contrary, radioautography can only demonstrate the newly synthesized macromolecules such as synthetic DNA or RNA or proteins depending upon the RI-labeled precursors incorporated specifically into these macromolecules such as ^{3}H-thymidine into DNA or ^{3}H-uridine into RNA or ^{3}H-amino acid into proteins (Nagata 2002).

Concerning to the newly synthesized macromolecules, the results of recent studies in our laboratory by the present author and co-workers should be reviewed in this article according to the classification of macromolecules as follows.

1.2.1 The DNA synthesis

The DNA (deoxyribonucleic acid) contained in cells can be demonstrated either by morphological histochemical techniques staining tissue sections such as Feulgen reaction (Feulgen and Rossenbeck 1924) or by biochemical techniques homogenizing tissues and cells. To the contrary, the synthetic DNA or newly synthesized DNA but not all the DNA can be detected as macromolecular synthesis together with other macromolecules such as RNA or proteins in various organs of experimental animals by either morphological or biochemical procedures employing RI-labeled precursors. We have studied the sites of macromolecular synthesis in almost all the organs of mice during their aging from prenatal to postnatal development to senescence by means of microscopic radioautography, one of the morphological methods (Nagata 1992, 1994b,c,d, 1996a,b,c,d, 1997a, 2002, 2010c). The results should be here described according to the order of organ systems in anatomy or histology.

1.2.2 The RNA synthesis

The RNA (ribonucleic acid) contained in cells can be demonstrated either by morphological histochemical techniques staining tissue sections such as methyl green-pyronin staining or by biochemical techniques homogenizing tissues and cells. To the contrary, the synthetic RNA or newly synthesized RNA but not all the RNA in the cells can be detected as macromolecular synthesis together with other macromolecules such as DNA or proteins in various organs of experimental animals by either morphological or biochemical procedures employing RI-labeled precursors. We have studied the sites of macromolecular synthesis in almost all the organs of mice during their aging from prenatal to postnatal development to senescence by means of microscopic radioautography, one of the morphological methods (Nagata 1992, 1994b,c, 1996a,b,c, 1997a, 2002, 2010c). The results obtained from RNA synthesis should be here described according to the order of organ systems in anatomy or

histology. In contrast to the results obtained from DNA synthesis of almost all the organs, we have studied only several parts of the organ systems with regards RNA. The skeletal system, the muscular system or the circulatory system were not so much studied.

1.2.3 The protein synthesis

The proteins found in animal cells are composed of various amino-acids which initially form low molecular polypeptides and finally macromolecular compounds designated as proteins. They are chemically classified into two, simple proteins and conjugated proteins. Therefore, the proteins can be demonstrated by showing specific reactions to respective amino-acids composing any proteins. Thus, the proteins contained in cells can be demonstrated either by morphological histochemical techniques staining tissue sections such as Millon reaction (Millon 1849) or tetrazonium reaction or otherwise by biochemical techniques homogenizing tissues and cells. To the contrary, the newly synthesized proteins but not all the proteins in the cells can be detected as macromolecular synthesis together with other macromolecules such as DNA or RNA in various organs of experimental animals by either morphological or biochemical procedures employing RI-labeled precursors. We have studied the sites of macromolecular synthesis in almost all the organs of mice during their aging from prenatal to postnatal development to senescence by means of microscopic radioautography (Nagata 1992, 1994b,c, 1996a,b, 1997a, 2002, 2010c). The results obtained from protein synthesis should be described according to the order of organ systems in anatomy or histology. In contrast to the results obtained from DNA synthesis of almost all the organs, we have studied only several parts of the organ systems.

1.2.4 The glucide synthesis

The glucides found in animal cells and tissues are composed of various low molecular sugars such as glucose or fructose called monosaccharides which form compounds of polysaccharides or complex mucopolysaccharides connecting to sulfated compounds. The former are called simple polysaccharides, while the latter mucopolysubstances. Thus, the glucides are chemically classified into 3 groups, monosaccharides such as glucose or fructose, disaccharides such as sucrose and polysaccharides such as mucosubstances. However, in most animal cells polysaccharides are much more found than monosaccharides or disaccharides. The polysaccharides can be classified into 2, i.e. simple polyscaccharides and mucosubstances. Anyway, they are composed of various low molecular sugars that can be demonstrated by either histochemical reactions or biochemical techniques. To the contrary, the newly synthesized glucides but not all the glucides in the cells and tissues can be detected as macromolecular synthesis together with other macromolecules such as DNA, RNA or proteins in various organs of experimental animals by either morphological or biochemical procedures employing RI-labeled precursors. We have studied the sites of macromolecular synthesis in almost all the organs of mice during their aging from prenatal to postnatal development to senescence by means of microscopic radioautography (Nagata 1992, 1994a,b,c, 1996a, 1997a, 2002, 2010c). The results obtained from glucides synthesis are described according to the order of organ systems in anatomy or histology. In contrast to the results obtained from DNA synthesis of almost all the organs, we have studied only several parts of the organ systems. The skeletal system, the muscular system and the circulatory system were not yet studied.

1.2.5 The lipid synthesis

The lipids found in animal cells are chemically composed of various low molecular fatty acids. They are esters of high fatty acids and glycerol that can biochemically be classified into simple lipids and compound lipids such as phospholipids, glycolipids or proteolipids. The simple lipids are composed of only fatty acids and glycerol, while the latter composed of lipids and other components such as phosphates, glucides or proteins. In order to demonstrate intracellular localization of total lipids, we can employ either histochemical reactions or biochemical techniques. To the contrary, the newly synthesized lipids but not all of the lipids in the cells can be detected as macromolecular synthesis similarly to the other macromolecules such as DNA, RNA, proteins or glucides in various organs of experimental animals by either morphological or biochemical procedures employing RI-labeled precursors. We have studied the sites of macromolecular synthesis in almost all the organs of mice during their aging from prenatal to postnatal development to senescence by means of microscopic radioautography (Nagata 1992, 1994a,b,c,d,e, 1996a, 1997a, 2002, 2010c). However, we have not studied the lipids synthesis so much as compared to other compounds. We have studied only a few organs of the digestive system.

1.3 The intracellular localization of the other substances

The other substances than macromolecules that can also be demonstrated by radioautography are target tracers not the precursors for the macromolecular synthesis. They are hormones such as ^{3}H-methyl prednisolone (Nagata et al. 1978b), neurotransmitters and inhibitors such as ^{14}C-bupranolol, a beta-blocking agent (Tsukahara et al. 1980) or ^{3}H-befunolol (Nagata and Yamabayashi 1983, Yamabayashi et al. 1981), vitamins, drugs such as synthetic anti-allergic agent ^{3}H-tranilast (Nagata et al. 1986b, Nishigaki et al. 1987, 1990a.b, Momose et al. 1989), hypolipidemic agent bezafibrate (Momose et al. 1993a,b, 1995), calmodulin antagonist (Ohno et al. 1982, 1983) or anti-hypertensive agent ^{3}H-benidipine hydrochloride (Suzuki et al. 1994), toxins, inorganic substances such as mercury (Nagata et al. 1977b) and others such as laser beam irradiation (Nagata 1984). The details are referred to the previous publication on the radioatuographology (Nagata 2002). However, their relationships to the cell aging and senescence were not studied.

1.4 Macromolecular synthesis in the normal organ systems

With regards to the macromolecular synthesis such as DNA, RNA, proteins, glucides, lipids etc in various cells and tissues, we have studied various cells and tissues in almost all the organ systems in the experimental animals such as mice and rats. Therefore, the results are classified into the organ systems in anatomy and histology, i.e. the organ of movement including the skeletal system and the muscular system, the circulatory organs, the digestive organs, the respiratory organs, the urinary organs, the reproductive organs, the endocrine organs, the nervous system, and the sensory organs. Thus, the results should be described according to this order in the following chapters divided into 4 chapters.

1.5 Macromolecular synthesis in the tumor cells

As for the tumor cells, on the other hand, which do not belong to any organ systems of the normal organs but grow in any organ systems, the macromolecular synthesis in the tumor

cells should be described separately from the normal organ system at the end of this book in chapter 4.

2. Macromolecular synthesis in the organ of movement

The Organ of Movement or locomotive organ of men and experimental animals consists of both the skeletal system and the muscular system. The former consists of many bones, around 200 in case of men while the latter consists of many skeletal muscles around 600 in case of men. We studied the macromolecular synthesis in a part of these locomotive organs in the experimental animals, but not all of them. The results should be described in the following 2 sections, the skeletal system and the muscular system.

2.1 Macromolecular synthesis in the skeletal system

The skeletal system of men and experimental animals consists of bones, joints and ligaments. We studied the DNA synthetic activities of the bones and joints of experimental animals in development and aging (Kobayashi and Nagata 1994, Nagata 1998c).

2.1.1 The DNA synthesis in the skeletal system

We studied the DNA synthetic activities of the bones and joints of experimental animals in development and aging to senescence (Kobayashi and Nagata 1994, Nagata 1998c).

2.1.1.1 The DNA synthesis in the bone

We studied the ossifications of salamander skeletons from hatching to senescence (Nagata 1998c). The fore-limbs (Fig. 2A) and hind-limbs (Fig. 2B) of salamanders were composed of skeletons consisting of bones and cartilages which were covered with skeletal muscles, connective tissues and epidermis consisting of stratified squamous epithelial cells in the outermost layer. The bones of juvenile salamanders at 4 weeks consisted of the hyaline cartilage (Figs. 2A, 2B). The hyaline cartilage consisted of spherical or polygonal cartilage cells or chondrocytes at the center. They were surrounded by rich interstitial ground substance which stained deep blue with toluidine blue staining. The spherical cartilage cells at the center of the bone changed their shapes to flattened shape under the perichondrium or free joint surfaces. Some of the nuclei of the chondrocytes were covered with silver grains when labeled with ^{3}H-thymidine (Figs. 2A, 2B). Mitotic figures were frequently seen in spherical cartilage cells in young animals. Examination of radioautograms at the young stages such as 4 weeks after hatching showed that many spherical cartilage cells and flattened cartilage cells were predominantly labeled. At 6 weeks after hatching, the size of bones enlarged and the number of cartilage cells increased. At this stage, however, the number of labeled cells in the cartilage cells in both fore-limbs (Fig. 2C) and hind-limbs (Fig. 2D) decreased as compared with the previous stage (Figs. 2A, 2B). The size of bones in juvenile animals at 8, 9, 10, and 11 weeks enlarged gradually (Fig. 2E, 2F). Radioautograms at these stages showed that the number of the labeled cells remarkably reduced as compared with those of 4 and 6 weeks. In the adult salamanders at 8 months up to 12 months, the bones showed complete mature structure and examination of radioautograms revealed that the number of labeled cells reached almost zero (Kobayashi and Nagata 1994, Nagata 2006c). No difference was found on the morphology and labeling between the fore-limbs and hind-limbs at any stage.

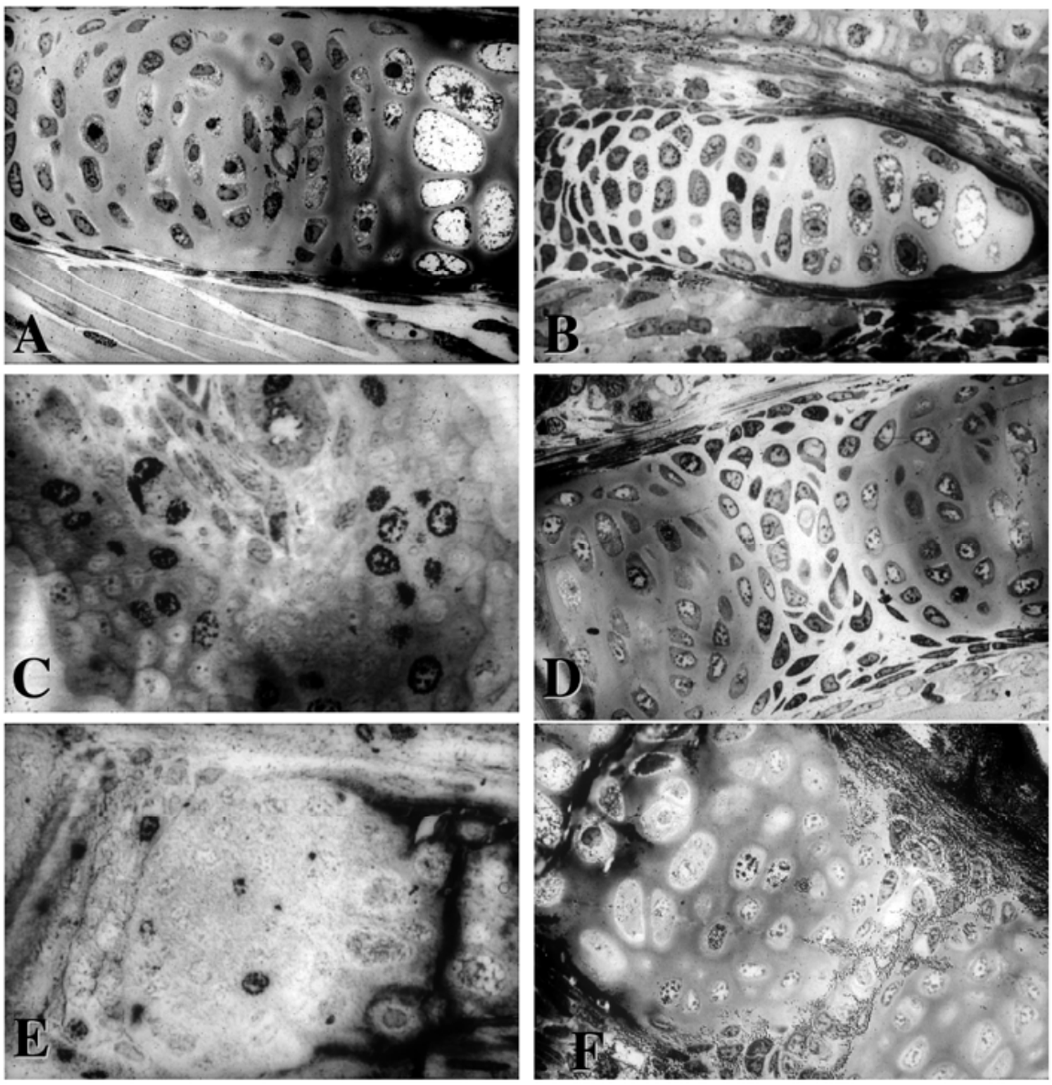


Fig. 2. Light microscopic radioautograms of the bones of either fore-limbs or hind-limbs of salamanders at various ages from 4 weeks to 8 weeks after hatching, injected with ^{3}H-thymidine, fixed and processed for radioautography. Some of the cartilage cells (arrows) are labeled with silver grains due to ^{3}H-thymidine incorporation demonstrating DNA synthesis. From Nagata, T.: Bulletin Shinshu Inst. Alternat. Med. Vol. 2, p. 53, 2006, Nagano, Japan
Fig. 2A. Light microscopic radioautogram of the bone of a fore-limb of a salamander at 4 weeks after hatching. Many cartilage cells (arrows) are labeled with silver grains due to ^{3}H-thymidine. Magnification. x 1200.
Fig. 2B. Light microscopic radioautogram of the bone of a hind-limb of a salamander at 4 weeks after hatching. Many cartilage cells (arrows) are labeled with silver grains due to ^{3}H-thymidine. Magnification. x 1200.
Fig. 2C. Light microscopic radioautogram of the bone of a fore-limb of a salamander at 6 weeks after hatching. Only a few cartilage cells (arrow) are labeled. The numbers of labeled cells are fewer than the bone of a younger salamander at 4 weeks after hatching (Fig. 1A). Magnification x 1200.

Fig. 2D. Light microscopic radioautogram of the bone of a hind-limb of a salamander at 6 weeks after hatching. Only a few cartilage cells (arrow) are labeled. Magnification x 1200.
Fig. 2E. Light microscopic radioautogram of the bone of a fore-limb of a salamander at 8 weeks after hatching. Only a few cartilage cells (arrow) are labeled. Magnification x 1200.
Fig. 2F. Light microscopic radioautogram of the bone of a hind-limb of a salamander at 8 weeks after hatching. Only a few cartilage cells (arrow) are labeled. Magnification x 1200.

The labeling indices of respective cell types changed with aging as expressed by mean in each group. The labeling index of the cartilage cells was lower than the epithelial cells. The peak of the labeling index of the cartilage cells in both fore-limbs and hind-limbs was found about 15-18% at 4 weeks after hatching (Fig. 2). The labeling index of the cartilage cells in both limbs at 6 weeks rapidly decreased to about 4-6%, then increased at 8 weeks to about 7-8% and finally decreased to 2-3% gradually from 8 weeks to 9 weeks with aging and fell down to 0-1% at 10 weeks. The labeling index of cartilage cells from 10 weeks to 12 months kept very low around 0-1% (Fig. 3). Thus, the cartilages and bones of fore-limbs and hind-limbs of salamanders are demonstrated to complete the development by 10 weeks after hatching (Kobayashi and Nagata 1994, Nagata 2006c).

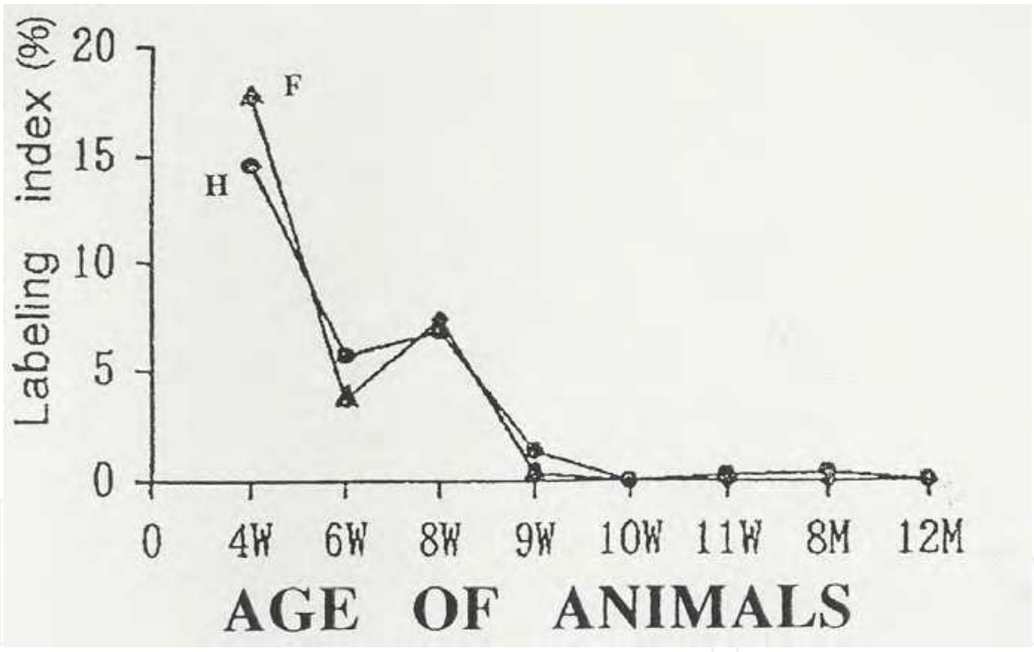


Fig. 3. Transitional curves of the labeling indices of the cartilage cells in the bones of the fore-limbs and the hind-limbs of salamanders labeled with ^{3}H-thymidine at various ages from 4 weeks to 60 months (5 years) after hatching. Mean ± S.D. From Nagata, T.: Bulletin Shinshu Inst. Alternat. Med. Vol. 2, p. 54, 2006, Nagano, Japan,

2.1.1.2 The DNA synthesis in the joint

The joints of an experimental animal such as mouse or human being are consisted of either 2 or 3 bones and the synovial membranes covering the ends of the bones. The synovial membranes are composed of the collagenous fibers interspersed with the synovial cells which are fibroblasts and lining cells. We studied macromolecular synthesis, both DNA and

RNA syntheses of the synovial cells of the joints surgically obtained from 15 elderly human patients of both sexes aged from 50 to 70, suffering from rheumatoid arthritis (Kobayashi and Nagata 1994). Both the normal and rheumatoid cells were cultured and labeled in vitro with media containing precursors such as ^{3}H-thymidine or ^{3}H-uridine, fixed and radioautographed. DNA synthetic cells labeled with silver grains were observed by LM RAG (light microscopic radioautography) in both normal and rheumatoid cells. As the results, some labeled synovial cells with ^{3}H-thymidine were found. However, no significant difference was observed between the labeling indices of normal and rheumatoid cells labeled with ^{3}H-thymidine. From the results, it was concluded that the synovial cells synthesized DNA in both normal and rheumatoid conditions. However, the quantities of these macromolecules synthesized in these synovial cells varied in respective individuals and no significant difference was found between the labeling indices and grain counts in both normal and rheumatoid cells (Kobayashi and Nagata 1994).

2.2 Macromolecular synthesis in the muscular system

The muscular system consists of various skeletal muscles amounting to around 600 in number in men and less in experimental animals such as rats and mice. We studied the aging changes of DNA synthesis in the intercostal muscles of aging ddY mice from prenatal day 13 through postnatal 24 months by ^{3}H-thymidine RAG (Hayashi et al. 1993).

2.2.1 The DNA synthesis in the muscular system

We studied the aging changes of DNA synthesis in the intercostal muscles of aging ddY mice from prenatal day 13 through postnatal 24 months in senescence by ^{3}H-thymidine RAG (Hayashi et al. 1993). Many nuclei were labeled in myotubes at embryonic day 13-17 (Fig. 4C) during development, then the number of labeled nuclei decreased to embryonic day 18-19 (Fig. 4D), and less due to aging after birth.

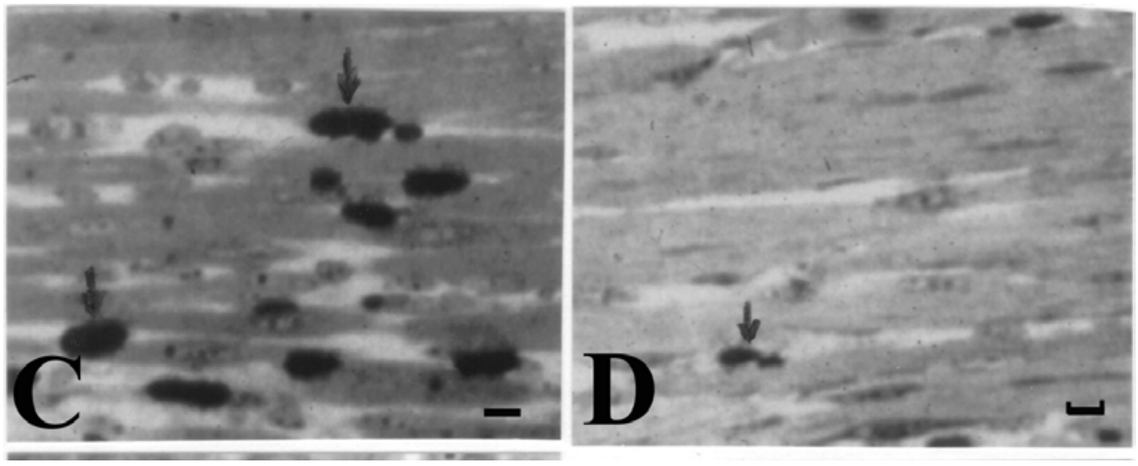


Fig. 4. Light microscopic radioautograms of the skeletal muscle cells in the myotubes labeled with ^{3}H-thymidine at embryonic day 13-17 (Fig. 3C), then the number of labeled nuclei decreased to embryonic day 18-19 (Fig. 3D), and less after birth. x260. From Nagata, T.: Special Cytochemistry in Cell Biology, In, Internat. Rev. Cytol. Vol. 211, No. 1, p. 62, 2001, Academic Press, San Diego, USA, London, UK.

The labeling indices revealed chronological changes, showing a peak at embryonic day 13 and decreasing gradually to 0% at 3 months after birth to month 24 (Fig. 5). We classified the graduation of the embryonic muscle development into 5 stages. Among them, the labeling index (LI) at stage I was the highest, while the LI at stage II was significantly lower than stage I, the LI at stage IV was significantly higher than stage II, and the LI at stage V was significantly lower than stage IV (Fig. 5). These changes accorded well with the primary and secondary myotube formation during the embryonic muscle development. We also studied the DNA synthesis of rat thigh muscles during the muscle regeneration after injury in rats (Sakai et al. 1977). When the skeletal muscles, i.e., the diaphragma, the rectus abdominis muscles and the gastrocnemius femoris muscle of adult Wistar rats were mechanically injured and labeled with ^{3}H-thymidine, satellite cells were labeled during their regeneration. The satellite cells in the muscles of dystrophy chickens and normal control chickens were also labeled with ^{3}H-thymidine, demonstrating DNA synthesis (Oguchi and Nagata 1980, 1981), which was later described in details in the review (Nagata 2002). Briefly, 2 groups of chickens, 4 dystrophy chickens and normal control chickens of both sexes aged 1 day and 21 days after hatching were used. All the animals received every 6 hrs intraperitoneal injections of ^{3}H-thymidine 4 times successively and sacrificed. The superficial pectoral muscles were taken out, fixed, embedded in Epoxy resin and processed for LM and EMRAG. The results demonstrated that many nuclei of the satellite cells in all the experimental groups were labeled but none of the nuclei in the muscle fibers were labeled. The labeling indices of normal chickens at 1 day and 21 days were 4.59 and 3.86%, respectively. These results showed that the LI decreased after hatching due to aging.

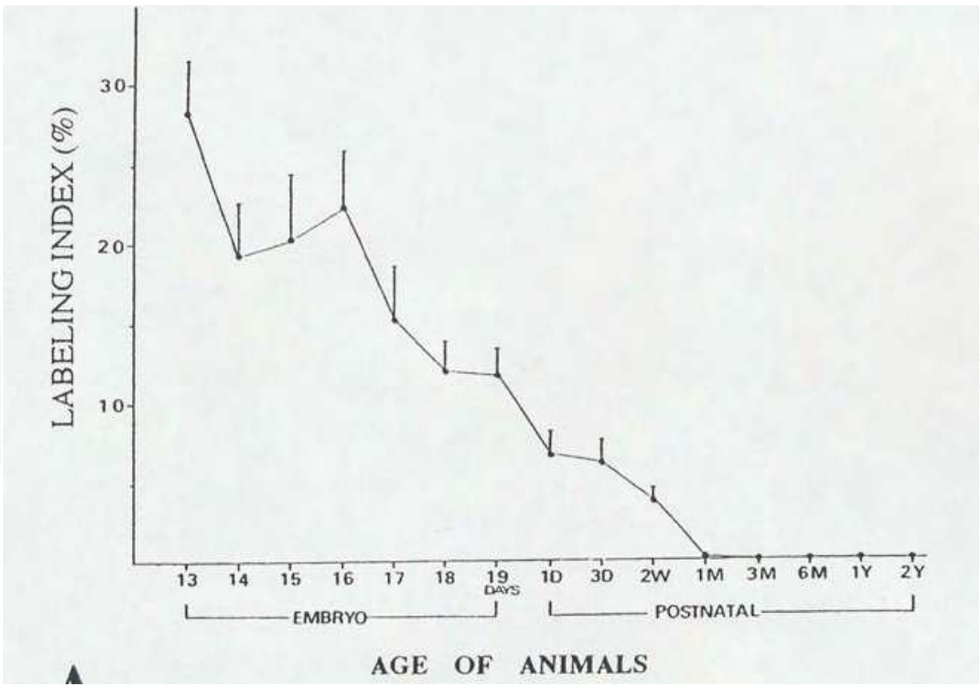


Fig. 5. The labeling indices of the muscular cells labeled with ^{3}H-thymidine revealed chronological changes, showing a peak at embryonic day 13 and decreasing gradually to 0% at 3 months after birth. From Nagata, T.: Radioautographology, General and Special. In, Prog. Histochem. Cytochem. Vol. 37, No. 2, p. 114, 2002, Urban & Fischer, Jena, Germany

2.2.2 The protein synthesis in the muscular system

We studied only ^{3}H-taurin incorporation in the skeletal muscles of both normal and dystrophy adult mice incubated in Eagle's medium containing ^{3}H-taurin in vitro at varying time intervals from 1 min to 5, 10, 30 and 60 min (1 hour). The silver grains were observed over the skeletal muscle cells as well as over the smooth muscle cells and the endothelial cells in the arteries, both the nuclei and cytoplalsm, by LM and EM RAG, showing taurin incorporation into the proteins (Terauch et al. 1988, Terauch and Nagata 1993, 1994). However, the aging changes were not studied yet.

3. Macromolecular synthesis in the circulatory system

The circulatory system or cardiovascular organs consists of the heart, the arteries, the veins, the capillaries, the blood, the lymphatic organs and the spleen. Among these cardiovascular organs, we studied the heart, the artery, some blood cells and the spleen.

3.1 The nucleic acid synthesis in the heart

Among the macromolecular synthesis, the nucleic acid synthesis, both DNA and RNA, in cultured cells from the hearts of chick embryos was studied by LM RAG (Nagata and Nawa 1966a,b). The fibroblasts of chick hearts in culture proliferated extensively and produced many binucleate cells. We compared the nucleic acid synthesis in mononucleate cells and binucleate cells in the heart fibroblasts. The incorporation of ^{3}H-thymidine into each nucleus of a binucleate cell was a little less than that of a mononucleate cell, but the total of the two nuclei of a binucleate cell was almost twice as that of a mononucleate cell. The incorporation of ^{3}H-uridine in the two nuclei of a binucleate cell was almost twice as that of a mononucleate cell, while the incorporation of ^{3}H-uridine in the cytoplasm of a binucleate cell was not so much as twice as a mononucleate cell. From these results, it was concluded that the nucleic acid synthesis both DNA and RNA increased in binucleate cells than mononucleate cells of chick embryo heart fibroblasts (Nagata and Nawa 1966a,b).

3.2 Localization of drugs in the artery

The structure of the blood vessels, both arteries and veins consist of 3 layers, from inside to outside, the tunica intima, the media and the adventitia. Those layers are formed with connective tissues and the smooth muscles. We studied the localization of anti-hypertensive drugs in the supramesenteric arteries of the spontaneous hypertensive rats (Suzuki et al. 1994). Two kinds of anti-hypertensive drugs, labeled with RI, ^{3}H-benidipine hydrochloride (Kyowa Hakko Kogyo Co., Shizuoka, Japan) and ^{3}H-nitrendipine (New England Nuclear, Boston, MA, USA) were used. Both intravenous administrations into rats and in vitro incubation for 10 to 30 min were employed. For light and electron microscopic radioautography, both the conventional wet-mounting radioautograms after chemical fixation for insoluble compounds and the dry-mounting radioautograms after cryo-fixation and freeze-substitution for soluble compounds were prepared. The silver grains due to the anti-hypertensive drugs were localized over the plasma membranes and the cytoplasm of the fibrocytes in the intima and the smooth muscle cells in the media, suggesting the pharmacological active sites. However, the localization of synthetic DNA was not studied.

3.3 The DNA synthesis in the blood cells

The mature blood cells circulating in the blood vessels of mammals are classified into 3 types, the erythrocytes, the leukocytes and the blood platelets. Those mature cells are formed either in the lymphatic tissues in the lymphatic organs or the myeloid tissues in the bone marrow, where various immature cells, lymphoblasts, erythroblasts, myeloblasts, meylocytes, and megakaryocytes can be observed. Among these blood cells, we studied macromolecular synthesis and cytochemical localization in leukocytes, megakaryocytes and blood platelets. As for the granulocytes, normal rabbit granulocytes were shown by EM RAG and X-ray microanalysis to incorporate $^{35}SO_4$ into the Golgi apparatus and to the granules demonstrating glucosaminoglycan synthesis (Murata et al. 1978, 1979).

On the other hand, the DNA, RNA and mucosubstance synthesis of mast cells from Wistar strain rats were studied by ^{3}H-thymidine, ^{3}H-uridine and $^{35}SO_4$ radioautography, demonstrating incorporation changes of those normal mast cells from abnormal mastocytoma cells (Murata et al. 1977a). Mast cells were widely found distributing in the loose connective tissues of most mammals, as well as in the serous exudate in the peritoneal cavity as one of the free cells. We studied the fine structure and nucleic acid and mucosubstance syntheses of normal mast cells and Dunn and Potter's mastocytoma cells in mice and rats by electron microscopic radioautography (Murata et al. 1977b, 1979). As the results, some of the normal mast cells and mastocytoma cells incorporated ^{3}H-thymidine, ^{3}H-uridine and $^{35}SO_4$, demonstrating DNA, RNA and mucosubstance syntheses. The incorporation of ^{3}H-thymidine was observed in the nuclei and mitochondria. The labeling index of ^{3}H-thymidine incorporation in the nuclei and mitochondria of normal mast cells was very low (0.37%) while that of mastocytoma cells was high (2-5%). These results suggested that the macromolecular synthesis such as nucleic acids (DNA, RNA) and mucosubstances were higher in tumor cells than normal blood cells.

3.4 Macromolecular synthesis in the spleen

The spleen is one of the blood cell forming organs and is composed of the lymphatic tissues. Among the macromolecular synthesis, both the DNA, RNA and protein syntheses in the spleen were studied.

3.4.1 The DNA synthesis in the spleen

We studied ^{3}H-thymidine incorporation into the splenic cells of aging mice from newborn to adult and senescence in connection with the lysosomal acid phosphatase activity (Olea 1991, Olea and Nagata 1991, 1992a). The acid phosphatase activity as demonstrated by means of cerium substrate method was observed in the splenic tissues at various ages from postnatal day 1, week 1 and 2, month 1, 2 and 10. Electron dense deposits were localized in the lysosomes of macrophages, reticular cells and littoral cells in all the aging groups. The intensity of the reaction products as visually observed increased from day 1 to week 1, reaching the peak at 1 week, and decreased from week 2 to month 10 due to aging. The incorporation of ^{3}H-thymidine, on the other hand, demonstrating DNA synthesis, was mainly observed in the hematopoietic cells in the spleens from postnatal day 1 to month 10 animals (Olea and Nagata 1991, 1992a). The labeling index was the maximum at day 1 and decreased to week 1, 2, 4, 8 and 40. A correlation between DNA synthesis and AcPase activity was examined by comparing two cell populations in the cell cycle, the S-phase cells

which were labeled with ^{3}H-thymidine and the non-S-phase cells or the interphase cells which were not labeled. It was demonstrated that the former showed an increase and decrease of much more AcPase activity with the aging while the latter less activity and no change.

3.4.2 The RNA synthesis in the spleen

On the other hand, the number of labeled cells and the grain counts in the hematopoietic cells in the spleens labeled with ^{3}H-uridine, demonstrating RNA synthesis, from postnatal day 1 increased to 1 and 2 weeks, reaching the maximum, and decreased to 4, 8 and 40 weeks, different from the DNA synthesis (Olea and Nagata 1992b). These results demonstrated that AcPase activity, DNA and RNA synthetic activity changed due to aging.

3.4.3 The protein synthesis in the spleen

Among the circulatory organs, we first studied the protein synthesis in the spleens of aging mice at various ages. Several groups of litter mates, each 3, from fetal day 19 to postnatal day 1, 14, and month 6 to 12 (year 1) were administered with ^{3}H-leucine and sacrificed, the spleens were taken out and processed for LM and EM RAG (Nagata and Olea 1999). The results demonstrated that the sites of incorporations were hematopoietic cells, i.e., lymphoblasts, myeloblasts, erythroblasts and littoral cells in the splenic tissues at every aging stage. In most labeled cells silver grains were observed over the nuclei, nucleoli, endoplasmic reticulum, ribosomes, Golgi apparatus and mitochondria. Quantitative analysis revealed that grain counts in respective cells were higher in young animals than adult aged animals. The grain counts and the labeling index increased from prenatal to postnatal day 14, reaching the maximum, then decreased to month 12. These results showed the increase and decrease due to aging of animals.

4. Conclusion

This chapter deals with the introductory remarks describing the method and procedure of microscopic radioautography as well as the first parts of its application to the organ systems such as the skeletal, muscular and the circulatory organs. The method and the procedure of microscopic radioautography described the detailed technology which was developed by the present author and associates since 1955 in our laboratory. The application of radaioautography to the skeletal, muscular and circulatory systems demonstrated the sites of macromolecular syntheses such as DNA, RNA, proteins, glucides and lipids in various organs as well as the quantitative changes due to aging of the experimental animals. These results should be very important to understand the fundamental changes in the respective bones, the skeletal muscles, and the cardiovascular organs as well as the contributions to the experimental biology and medicine throughout the world.

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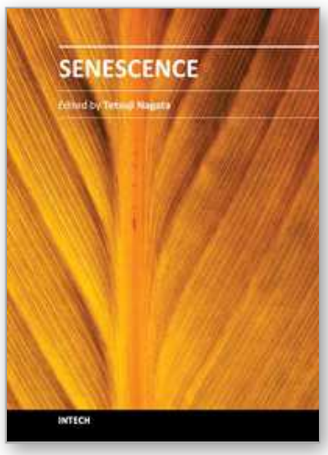
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