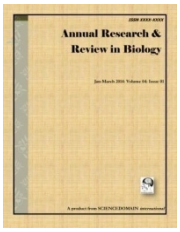

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Cell Aging of Mouse Liver as Observed By Microscopic Radioautography

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Author's contribution

This whole work was carried out by the author TN.

Original Research Article

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ABSTRACT

"Cell Ageing" means how the cells change due to their aging. The term contains 2 meanings, one how a cell changes when it is isolated from original animals such as in vitro cells in cell culture or tissue culture, while the other means how all the cells of an animal such as mice and rats changes in vivo due to the aging of the individual animal. Our group have been studying the latter changes from the viewpoint of the cell nutrients, the precursors for the macromolecular synthesis such as DNA, RNA, proteins, glucides and lipids, which are incorporated and synthesized into various cells, cell organelles both nuclei and cytoplasm of individual animals. Therefore, this article deals with only the cell aging of animal cells in vivo, how the metabolism, i.e., incorporations and syntheses of respective nutrient precursors in various kinds of cells change due to the ageing of individual experimental animals such as mice and rats by means of microscopic radioautography to localize the RI-labeled precursors. The incorporations and syntheses of various precursors for macromolecules 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 are already published from our laboratory during these 50 years since the late 20C to the early 21C. Among these publications, this paper deals with the cell ageing of the liver of mouse reviewing many papers already published from our laboratory.

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1. INTRODUCTION

The term “Cell Ageing” initially means how the cells change 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. Our group had first studied the meaning of cell aging 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 [1-3], and then moved to the study on the latter cell ageing, i.e., how all the cells of an experimental animal change in vivo due to the aging of the individual prenatal and postnatal animal [4-8].

Recently, our group have been studying the aging changes from the viewpoint of the cell nutrients which were incorporated and synthesized into various cells in individual animals during their aging [9]. Therefore, this article deals with only the cell aging 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 aging 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.

1.1 Radioautography

In order to observe the localizations of the incorporations and syntheses of various nutrients synthesizing macromolecules in the bodies such as DNA, RNA, proteins, glucides and lipids in various kinds of cells of various organs 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 [10].The macromolecules are classified into several categories such as nucleic acids (both DNA and RNA), proteins, glucides and lipids, according to their chemical structures.

The technique is designated as radioautography using RI-labeled compounds. 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 protein, ^{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 [11]. As the results, specimens prepared for EM RAG are very thick and should be observed with high voltage electron microscopes in order to obtain better transmittance and resolution [12,13]. Such radioautographic techniques in details should be

referred to other literature [14]. On the other hand, the systematic results obtained byradioautography should be designated as radioauographology, or science of radioautography [15-17]. This article deals with the results dealing with the radioautographic changes of individual cell by aging that should be included in radioautographology.

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 [18] that stains all DNA contained in the cells. Each compounds of macromolecules such as DNA, RNA, proteins, glucides, lipids can be demonstrated by respective specific histochemical stainings [19] and such reactions can be quantified by *microspectrophotometry* using specific wave-lengths demonstrating the total amount of respective compounds [20]. 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 protein [14].

Concerning to the newly synthesized macromolecules, the results of recent studies in our laboratory by the present author and co-workers using mice as experimental animals should be reviewed in this article according to the classification of macromolecules as follows. The experimental animals, pregnant ddY mice were purchased from Shizuoka Animal Co., Shizuoka, Japan, and the new born mice were grown up in our laboratory.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. All the procedures used in this study concerning the animal experiments were in accordance with the guidelines of the animal research committee of Shinshu University School of Medicine, Matsumoto, Japan, where this experiment was carried out, as well as the principles of laboratory animal care in NIH publication No. 86-23 (revised 1985).

2. 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 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 [21-29]. The results obtained from the digestive organs should be here described according to the order of the organ system in anatomy or histology.

2.1 The DNA Synthesis in the Digestive System

The digestive system anatomically consists of the digestive tract and the digestive glands. The digestive tract can be divided into several portions, the oral cavity, the pharynx, the

esophagus, the stomach, the small and large intestines and the anus, while the digestive glands consist of the large glands such as the salivary glands, the liver and the pancreas and the small glands affiliated to the digestive tracts in the gastrointestinal walls such as the gastric glands, intestinal glands of Lieberkuehn or duodenal glands of Brunner. We have published many papers from our laboratory dealing with the macromolecular synthesis of respective digestive organs from the oral cavity to the gastrointestinal tracts and the digestive glands [30-44,14]. The outline of the results concerning to the synthetic DNA in the digestive organs especially the liver should be here described in the order of systematic anatomy and special histology as follows.

2.1.1 The DNA synthesis in the oral cavity

The oral cavity consists of the lip, tongue, tooth, and the salivary gland. The DNA synthesis of mucosal epithelia of the 2 upper and lower lips and the tongues as well as the 3 large salivary glands and many small glands of aging mice from fetal day 19 to postnatal 2 years were studied by LM and EM RAG labeled with ^{3}H-thymidine. The glucide and glycoprotein syntheses by ^{3}H-glucosamine and radio-sulfate incorporations of the submandibular and sublingual glands of aging mice were also studied.

We first studied the DNA synthesis of the submandibular glands in 10 groups of aging mice at various ages from embryo to postnatal 2 years [40,44]. The submandibular gland of male mouse embryonic day 19 consisted with the glandular acini and duct system (Fig. 1A). The duct system was composed of juxtaacinar cells (JA), intercalated duct cells (ICD) and striated duct cells (SD). Many labeled developing acinar cells (AC), JA and ICD cells were observed. At postnatal day 1 to 3 (Fig. 1B), there was more JA cells and secretory granules than those of former stage. JA cells were cuboidal cells, characterized by small darkly stained granules in the supranuclear cytoplasm and by basophilic mitochondria mostly at the basal half of the cells. JA cells were present at the acinar-intercalated duct junction of the mouse submandibular gland. Many labeled AC, JA, ICD and SD cells were also observed by electron microscopy (Fig. 1C). At postnatal 2 weeks to 3 months, developing immature acinar cells gradually matured to acinar cells, and JA cells increased and granular convoluted duct cells (GCT) appeared. At postnatal 6 months to 2 years, the GCT cells were very well developed and were composed of the taller cells packed with many granules and became highly convoluted, and only a few labeled cells were found. The aging changes of frequency of 5 main individual cell types in submandibular glands of male mouse from embryonic day 19 to postnatal 2 years of age were counted. On embryonic day 19 of age, the gland consisted of developing acinar cells (49%), intercalated duct cells (37%), juxta-acinar (JA) cells (3%), striated duct (SD) cells (11%). At birth, JA cells increased rapidly to 32%, thereafter decreased gradually. At 1 month of age, JA cells disappeared and granular convoluted tubule (GCT) cells appeared and increased rapidly in number with age. They reached a maximum at 6 months. Then they decreased gradually from 6-21 months. The quantity proportion of acini was relatively stable during these periods. The frequency of ICD cells (Fig. 1C) was the highest (37%) at 1 day after birth. Thereafter it gradually decreased month by month and reached 2.6% at 21 months, while the ratio of SD cells persisted in 7%-12% from embryonic day 19 to postnatal 2 weeks and it disappeared at 3 months after birth. The proliferative activity of the cell population is expressed by the labeling index which is defined as the percentage of labeled nuclei with ^{3}H-thymidine in a given cell population. The labeling index of the entire gland cells increased from 13.6% at embryonic 19 to 18.3% at neonate, when it reached the first peak (Figs. 2A, 2B). Then it declined to 2.2% at 1 week of age. A second small peak (2.9%) occurred at 2 weeks. Thereafter, the labeling index decreased progressively to less than 1% at 4 weeks of age and then remained low. The

analysis of the labeling indices of respective cell types revealed that the first peak at neonate was due to the increased labeling indices of AC, ICD and JA cells, and the second peak at 2 weeks was due to the increase of ICD and SD cells. Thereafter, the labeling index of ICD cells decreased steadily but remained higher than those of any other cell types. Since the labeling index of ICD cells was more than the other cell types and persisted for a long time, it was suggested that ICD cells concerned with the generation of other cell types [14].

2.1.2 The DNA synthesis in the esophagus

The esophagus is the characteristic digestive tract including all the layers, the mucous membrane covered with the stratified squamous epithelia, the submucosa, the muscular layer and the serosa or adventitia. We studied the DNA synthesis of the esophagus of aging mice labeled with ^{3}H-thymidine by light microscopic and electron microscopic radioautography (LM and EM RAG) [45,46]. The labeled cells were mainly found in the basal layer of the esophageal epithelium (Fig. 1D). By electron microscopy the nuclei and nucleoli of labeled cells were larger than those of unlabeled cells, but contained fewer cell organelles [46]. The labeling indices in respective aging groups showed a peak at postnatal day 1 and decreased with aging keeping a constant level around a few % from 6 months to 2 years after birth.

2.1.3 The DNA synthesis in the stomach

The stomach consists of the mucosa covered with the surface epithelia of the columnar epithelia, including the gastric glands, the submucosa, the muscular layer and the serosa. As for the turnover of fundic glandular cells shown by ^{3}H-thymidine radioautography, it was extensively investigated with LM RAG by Leblond and co-workers [47,48,49]. They demonstrated that the DNA synthesis in the stomach increased at perinatal stages and decreased due to aging and senescence. However, the activity never reached zero but low activity continued until senescence. We studied the macromolecular synthesis including DNA, RNA, protein and glycoproteins in the gastric mucosa of both human and animal tissues by LM and EMRAG. As for the DNA synthesis, we obtained the same results as [49]. Therefore, the minute details will be here omitted.

2.1.4 The DNA synthesis in the intestines

The intestines of mammals are divided into 2 portions, small and large intestines, which can be further divided into several portions, the duodenum, the jejunum, the ileum, the caecum, the colon and the rectum. The intestinal tracts in any portions consist of the mucosa covered with columnar epithelial cells including absorptive and secretory cells, the submucosa, the smooth muscular layer and the serosa. We studied the macromolecular synthesis by LM and EMRAG mainly in the epithelial cells [14]. The DNA synthesis of small and large intestines of mice were studied by ^{3}H-thymidine RAG (Fig. 1E). The cells labeled with ^{3}H-thymidine were localized in the crypts of both small and large intestines, a region defined as the proliferative zone.

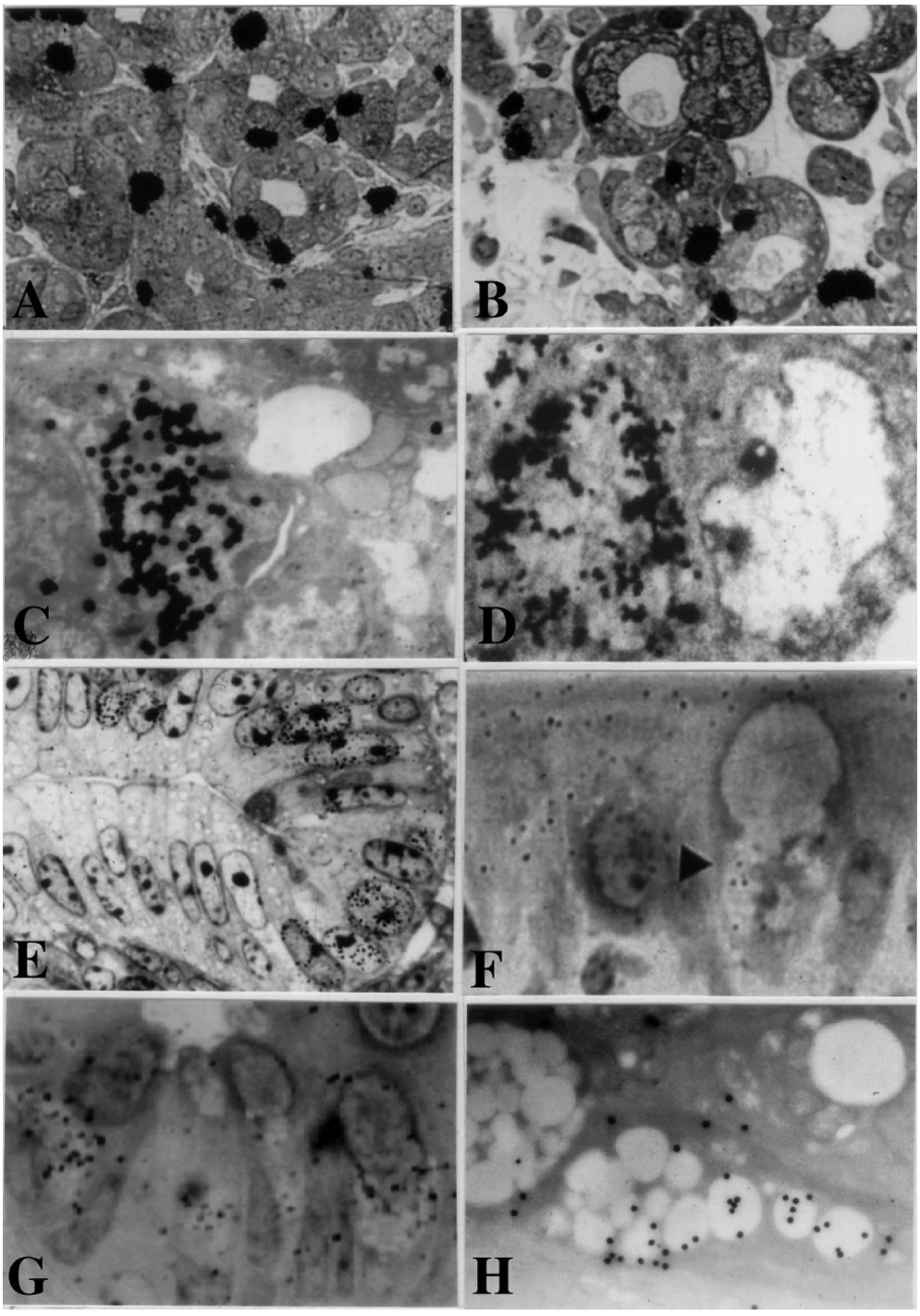

Fig. 1. LM and EM RAG of the digestive organs

Fig.1A. LMRAG of the submandibular gland of male mouse embryonic day 19 labeled with ^{3}H-thymidine consisted with the glandular acini and duct system. The duct system was composed of juxtaacinar cells (JA), intercalated duct cells (ICD) and striated duct cells (ICD). Many labeled developing acinar cells (AC), JA and ICD cells were observed. x500

Fig.1B. LMRAG of the submandibular gland at postnatal day 3, labeled with ^{3}H-thymidine. There were more JA cells and secretory granules than those of former stage (Figure 5A). x500
Fig.1C. EMRAG of an ICD cell of a mouse at postnatal day 3, labeled with ^{3}H-thymidine observed by electron microscopy. Many silver grains are observed over the nucleus of an ICD. x10,000
Fig.1D. EMRAG of the esophageal epithelial cells of a newborn mouse at postnatal day 1, labeled with ^{3}H-thymidine. Many silver grains are observed over one of the nuclei at left. x10,000
Fig.1E. LMRAG of the colonic epithelial cells of a mouse embryo at fetal day 19, labeled with ^{3}H-thymidine. Many silver grains are observed over the nuclei of several epithelial cells in the bottom of the crypt. x800
Fig.1F. LMRAG of the ileum epithelial cells labeled with ^{3}H-glucosamine of an old mouse at postnatal 6 months. Many silver gains are localized over the Golgi region of the 3 goblet cells as well as over the cytoplasm of several absorptive columnar epithelial cells. x 1,000
Fig.1G. LMRAG of the colonic epithelial cells of a mouse at postnatal month 1, labeled with $^{35}SO_4$ in vitro and radioautographed. x1,000
Fig.1H. EMRAG of a goblet cell in the deeper crypt of the colonic epithelial cells of an adult mouse after injection of $^{35}SO_4$ and radioautographed. Many silver grains are observed over the Golgi region and mucous droplets of the goblet cell, demonstrating the incorporation of radiosulfate into sulfomucins. x4,800
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In the colon of aging mice from fetal to postnatal 2 years, the labeled cells in the columnar epithelia were frequently found in the perinatal groups from embryo to postnatal day 1. However, the labeling indices became constant from the suckling period until senescence [50]. On the other hand, we examined the labeling indices of respective cell types in each layer of mouse colon such as columnar epithelial cells, lamina propria, lamina muscularis mucosae, tunica submucosa, inner circular muscle layer, outer longitudinal muscle layer, outer connective tissue and serous membrane of the colon and found that most labeling indices decreased after birth to 2 months except the epithelial cells which kept constant value to senescence (Fig. 3). Similar results were also obtained from the cecal tissues of mouse by LM and EMRAG. We also studied immunostaining for PCNA/cyclin and compared to the results obtained from RAG [50].

We fixed the colonic tissues of litter mice of six aging groups from the embryonic day 19, to newborn postnatal day 1, 5, 21, adult 2 months and senescent 12 months in methacarn and immunostained the colonic epithelium for cyclin proliferating nuclear antigen (PCNA/cyclin), which appeared from G1 to S phase of the cell cycle, with the monoclonal antibody and the avidin-biotin peroxidase complex technique. The immunostaining positive cells were localized in the crypts of colons similarly to the labeled cells with ^{3}H-thymidine by radioautography, a region defined as the proliferative zone. The positive cells in the columnar epithelia were frequently found in the perinatal groups from embryo to postnatal day 1, and became constant from postnatal day 5 until senescence. Comparing the results by immunostaining with the labeling index by radioautography, it was found that the former was higher in each aging group than the latter. The reason for the difference should be due to that PCNA/cyclin positive cells included not only S-phase cells but also the late G1 cells.

2.1.5 The DNA synthesis in the liver

The liver is the largest gland in the human and the mammalian body and consists of several types of cells [9]. The hepatocyte is the main component of the liver, composing the liver parenchyma which form the hepatic lobules, surrounded by other types of cells such as the connective tissue cells, sinusoidal endothelial cells, satellite cells of Kupffer, Ito's fat-storing cells and bile epithelial cells. In the livers of perinatal animals, the liver tissues include hematopoietic cells such as erythroblasts, myeloblasts and *megakaryocytes*.

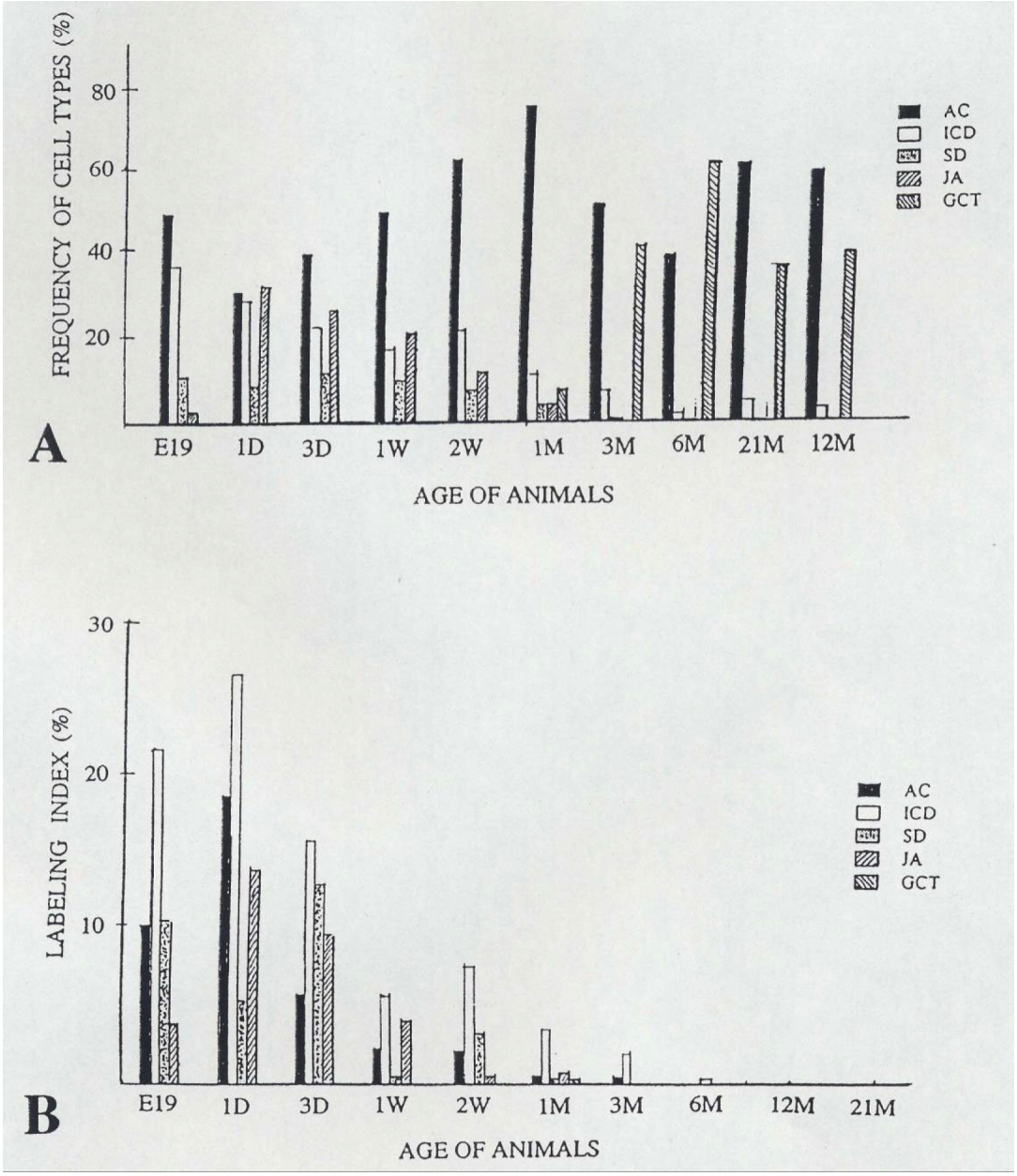

Fig. 2. Histogram showing the frequencies (A) and labeling indices (B) of the five individual cell types in the submandibular glands of male ddY mice at respective ages

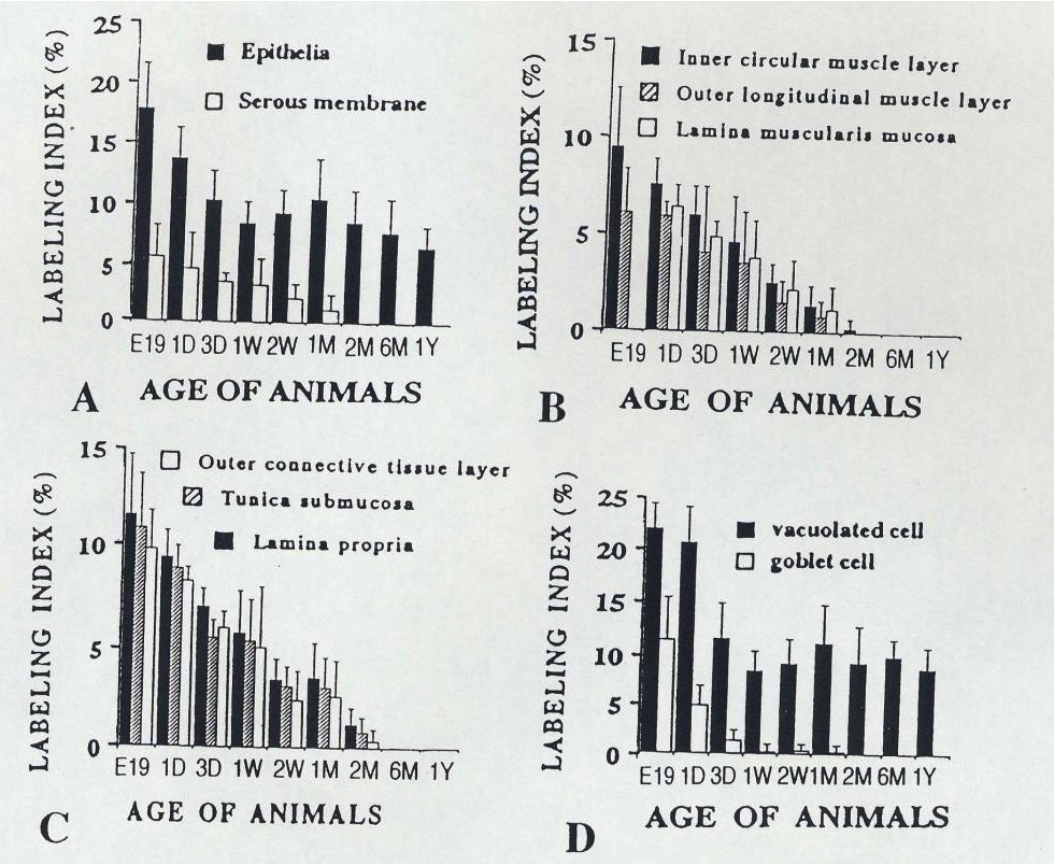

Fig. 3. Histogram showing aging changes of average labeling indices in respective tissue layers and cells of mouse colons at various ages from embryo to postnatal year 1, labeled with ^{3}H-thyidine

We first studied the liver tissues at various ages from embryo to postnatal 2 years [51,52,33,53,35,36,37,38,39,54,55,56,57,58,59,60,61,62,63,64,65,66,67,68,69,70].

The results obtained from the tissues of 3 groups of animals injected separately with 3 kinds of RI-labeled precursors, ^{3}H-thymidine, ^{3}H-uridine and ^{3}H-leucine were already reported as several original articles and reviews [71,72,73,74,75,76,77,78,79,80,81,82,83,84,85, 86,87,88,89,90,91,92,93,94] or as a monograph in the series of Prog. Histochem. Ccytochem [95,96,97]. Therefore, the results from the livers in aging mice are briefly summarized in this article.

2.1.5.1 The DNA synthesis in hepatocyte nuclei

We studied macromolecular synthesis by LM and EMRAG mainly in hepatocytes of rats and mice [22,23,24,33,34,35,36,37,38,55,69,85,86,88,89,98,99,100,101,102]. As for the nucleic acid synthesis, we first studied the difference between the mononucleate and binucleate hepatocytes of adult rats, injected with ^{3}H-thymidine and radioautographed [8,103]. The results showed that the frequency of labeled cells was greater in the mononucleate cells (Fig. 4A) than in the binucleate cells. The labeled binucleate cells were classified into two types,

i.e., a hepatocyte whose one of the two nuclei was labeled and a hepatocyte whose two nuclei were labeled. The former was more frequently observed than the latter. Grain counts revealed that the amount of DNA synthesized in the binucleate cell whose one nucleus was labeled was the same as the mononucleate cell, while the total amount of DNA synthesized in the binucleate cell whose two nuclei were labeled was almost twice as that of the mononucleate cell. These results suggest that the two nuclei of binucleate hepatocytes synthesize DNA independently from each other.

On the other hand, LM and EMRAG of prenatal and postnatal normal mice at various ages labeled with ^{3}H-thymidine revealed that many silver grains were localized over the nuclei of various cell types consisting the liver, i.e., hepatocytes (Fig. 4A), sinusoidal endothelial cells (Fig. 4B), Kupffer's cells, Ito's fat-storing cells, bile ductal epithelia cells, fibroblasts and hematopoietic cells [71,72,73,74,75,77,104]. In hematopoietic cells in the livers of perinatal animals, silver grains were observed over the nuclei of erythroblasts, myeloblasts, lymphoblasts and megekaryocytes. However, most hematopoietic cells disappeared on postnatal day 14. At fetal day 19, the liver tissues were chiefly consisted of hepatocytes and haematopoietic cells and no lobular orientation was observed. At postnatal day 1 and 3, lobular formation started and finally the hepatic lobules were formed at day 9 after birth. During the perinatal period, almost all kinds of cells were labeled with ^{3}H-thymidine. Percentage of labeled hepatocytes was the highest at fetal day 19, and rapidly decreased after birth to day 3. From day 9 to 14, percentage of labeled hepatocytes (labeling index) decreased gradually and finally to the lowest at 24 months (Fig. 5A). When the labeling indices of hepatocytes in 3 hepatic acinar zones were analyzed, the indices decreased in zone 2 (intermediate zone) and zone 3 (peripheral zone) on days 3 and 9 after birth, whereas they increased in zone 1 (central) on day 9, and then they altogether decreased from day 14 to 24 months (Fig. 5B). When the size and number of cell organelles in both labeled and unlabeled hepatocytes were estimated quantitatively by image analysis with an image analyzer, Digigramer G/A (Mutoh Kogyo Co. Ltd., Tokyo, Japan) on EMRAG, the area size of the cytoplasm nucleus, mitochondria, endoplasmic reticulum, and the number of mitochondria in the unlabeled hepatocytes were more than the labeled cells [104,105,106,107,108,109,110]. These data demonstrate that the cell organelles of the hepatocytes that synthesized DNA were not well developed as comparedto those not synthesizing DNA during the postnatal development. In some of unlabeled hepatocytes, several silver grains were occasionally observed localizing over mitochondria and peroxisomes as was formerly reported [111,112]. The mitochondrial DNA synthesis was first observed in cultured hepatocytes of chickens and mice in vitro [113,114] (Nagata et al. 1967a,b). The percentages of labeled cells in other cell types in the liver of aging mice such as sinusoidal endothelial cells, Kupffer's cells, Ito's fat-storing cells, bile ductal epithelia cells and fibroblasts showed also decreases from perinatal period to postnatal 24 months.

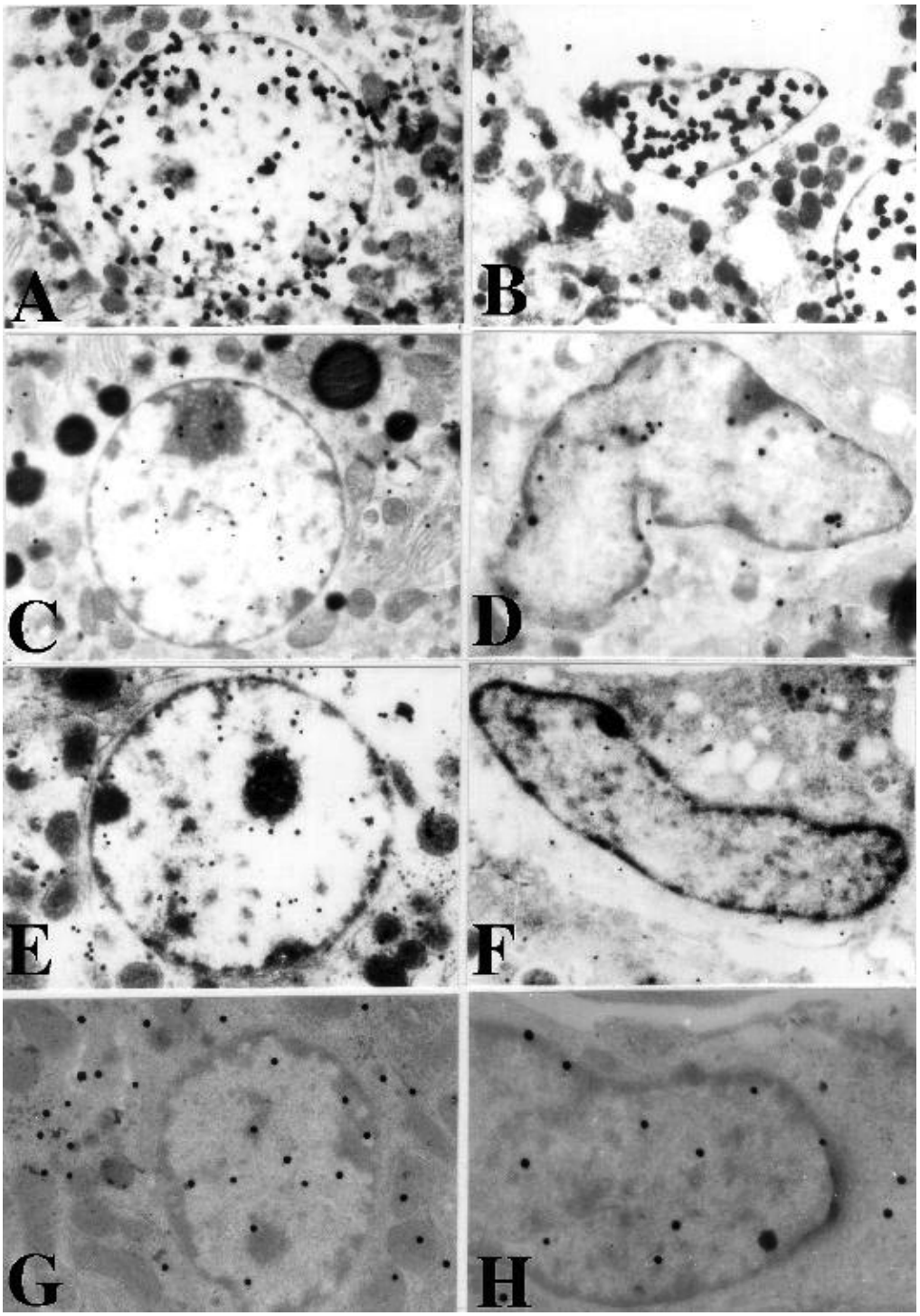

Fig. 4. EM RAG of the liver

Fig. 4A. EMRAG of a hepatocyte of the liver of a 14 day old mouse labeled with 3H-thymidine. Many silver grains were observed over the nucleus and mitochondria

Fig. 4B. EM RAG of a sinusoidal endothelial cells of the liver of a 14 day old mouse labeled with 3H-thymidine. Many silver grains were observed over the nucleus and mitochondria
Fig. 4C. EM RAG of a hepatocyte of the liver of a 14 day old mouse labeled with 3H-uridine. Many silver grains were observed over the nucleus and mitochondria
Fig. 4D. EM RAG of an Ito's fat-storing cell of the liver of a newborn 14 day old mouse labeled with 3H-uridine. Many silver grains were observed over the nucleus and mitochondria
Fig. 4E. EM RAG of a hepatocyte of the liver of a 1 month old mouse labeled with 3H-leucine. Many silver grains were observed over the nucleus and mitochondria
Fig. 4F. EM RAG of a sinusoidal endothelial cells of the liver of a newborn 14 day old mouse labeled with 3H-leucine. Many silver grains were observed over the nucleus and mitochondria.
Fig. 4G. EMRAG of a hepatocyte of the liver of an adult 2 month old mouse labeled with 3H-proline. Many silver grains were observed over the nucleus and mitochondria
Fig. 4H. EMRAG of a Kupffer cell of the liver of a newborn 1 day old mouse labeled with 3H-proline. Many silver grains were observed over the nucleus and mitochondria

2.1.5.2 Mitochondrial DNA synthesis in hepatocytes

When we observed DNA synthesis in the nuclei of mononucleate and binucleate hepatocytes, we also observed DNA synthesis in hepatocyte mitochondria [71,72,73,74,75,115,116]. The results of visual grain counts on the number of mitochondria labeled with silver grains obtained from 10 mononucleate hepatocytes of each animal labeled with ^{3}H-thymidine demonstrating DNA synthesis in 7 aging groups at perinatal stages, prenatal embryo day 19, postnatal day 3, 9 and 14, month 1, 6, 12 and 24, were counted. The number of total mitochondria per cell increased from perinatal stage (35-50/cell) increased to postnatal month 6 (95-105/cell), reaching the maximum, decreased to month 24 (85-90/cell), while the number of labeled mitochondria per cell increased from perinatal stage to postnatal day 14, reaching the maximum, decreased to month 6, then increased again to month 12, reaching the second peak and decreased again to month 24. Thus, the labeling indices in respective aging stages were calculated from the number of labeled mitochondria which showed an increase from perinatal stage to postnatal day 14, reaching the maximum and decreased to month 24. The results showed that thenumbers of labeled mitochondria with ^{3}H-thymidine showing DNA synthesis increased from prenatal embryo day 19 (3.8/cell) to postnatal day 14 (6.2/cell), reaching the maximum, and then decreased to month 6 (3.7/cell) and again increased to year 1 (6.0/cell), while the labeling indices increased from prenatal day 19 (11.8%) to postnatal day 14 (16.9%), reaching the maximum, then decreased to month 6 (4.1%) and year 1 (6.4%) and year 2 (2.3%). The increase of the total number of mitochondria in mononucleate hepatocytes was stochastically significant ($P<0.01$), while the changes of number of labeled mitochondria and labeling index in mononucleate hepatocytes were not significant ($P<0.01$).

As for the binucleate hepatocytes, on the other hand, because the appearances of binucleate hepatocytes showing silver grains in their nuclei demonstrating DNA synthesis were not so many in the adult and senescent stages from postnatal month 1 to 24, only binucleate cells at perinatal stages when reasonable numbers of labeled hepatocytes were found in respective groups were analyzed. The number of mitochondria in binucleate hepatocytes at postnatal day 1 to 14 kept around 80 (77-84/cell) which did not show such remarkable changes, neither increase nor decrease, as shown in mononucleate cells. Thus, the number of mitochondria per binucleate cell, the number of labeled mitochondria per binucleate cell and the labeling index of binucleate cell in 4 groups from postnatal day 1 to 14 were counted. The number of mitochondria and the number of labeled mitochondria were more in binucleate cells than mononucleate cells [115,116,117,118,119,120,121].

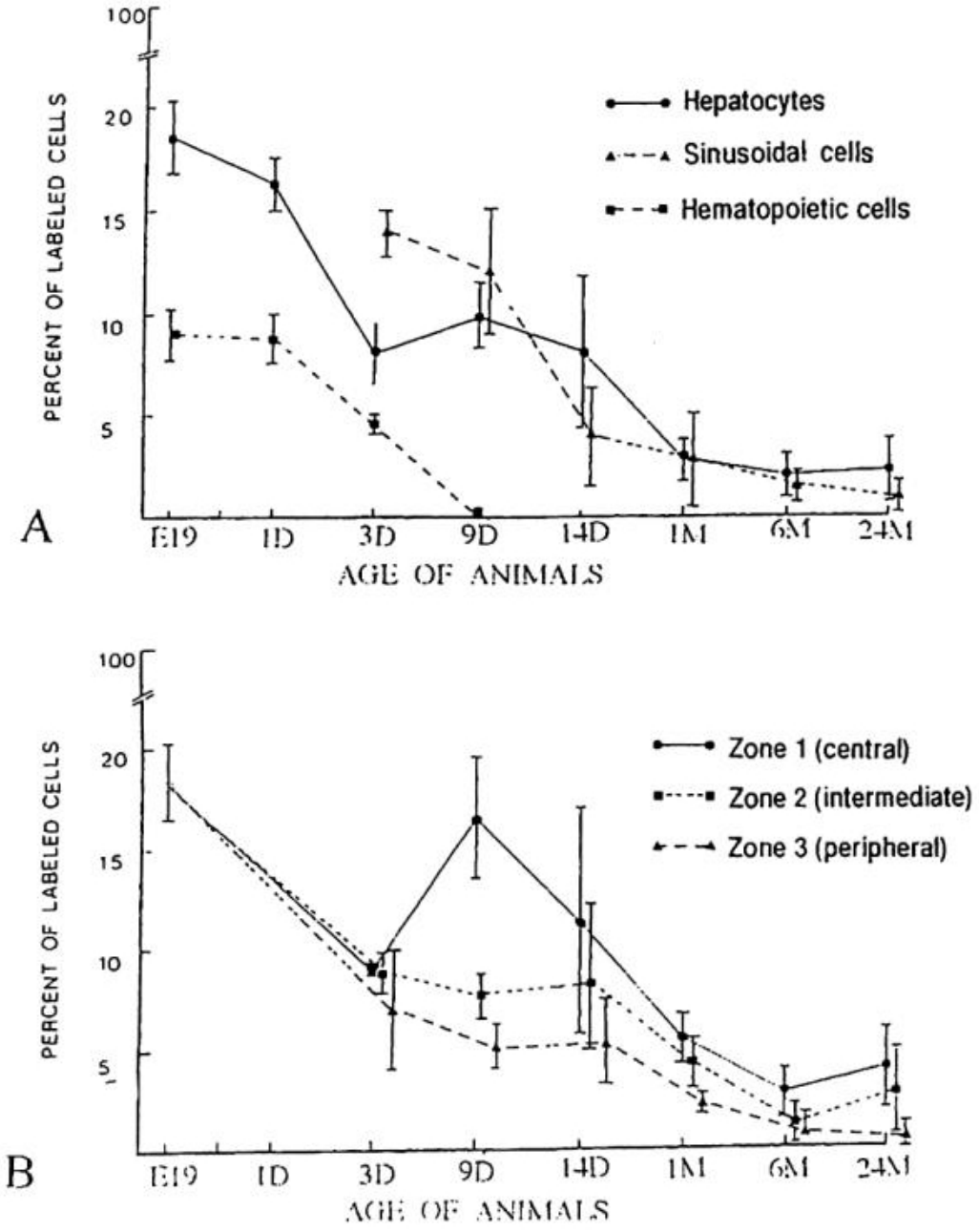

Fig. 5. Transitional curves of the labeling indices in the livers of aging mice after injection of ^{3}H-thymidine. Mean ± Standard Deviation

Fig. 5A. Labeling indices of hepatocytes, sinusoidal endothelial cells and hematopoietic cells, respectively

Fig. 5B. Labeling indices of hepatocytes, sinusoidal endothelial cells and hematopoietic cells, respectively

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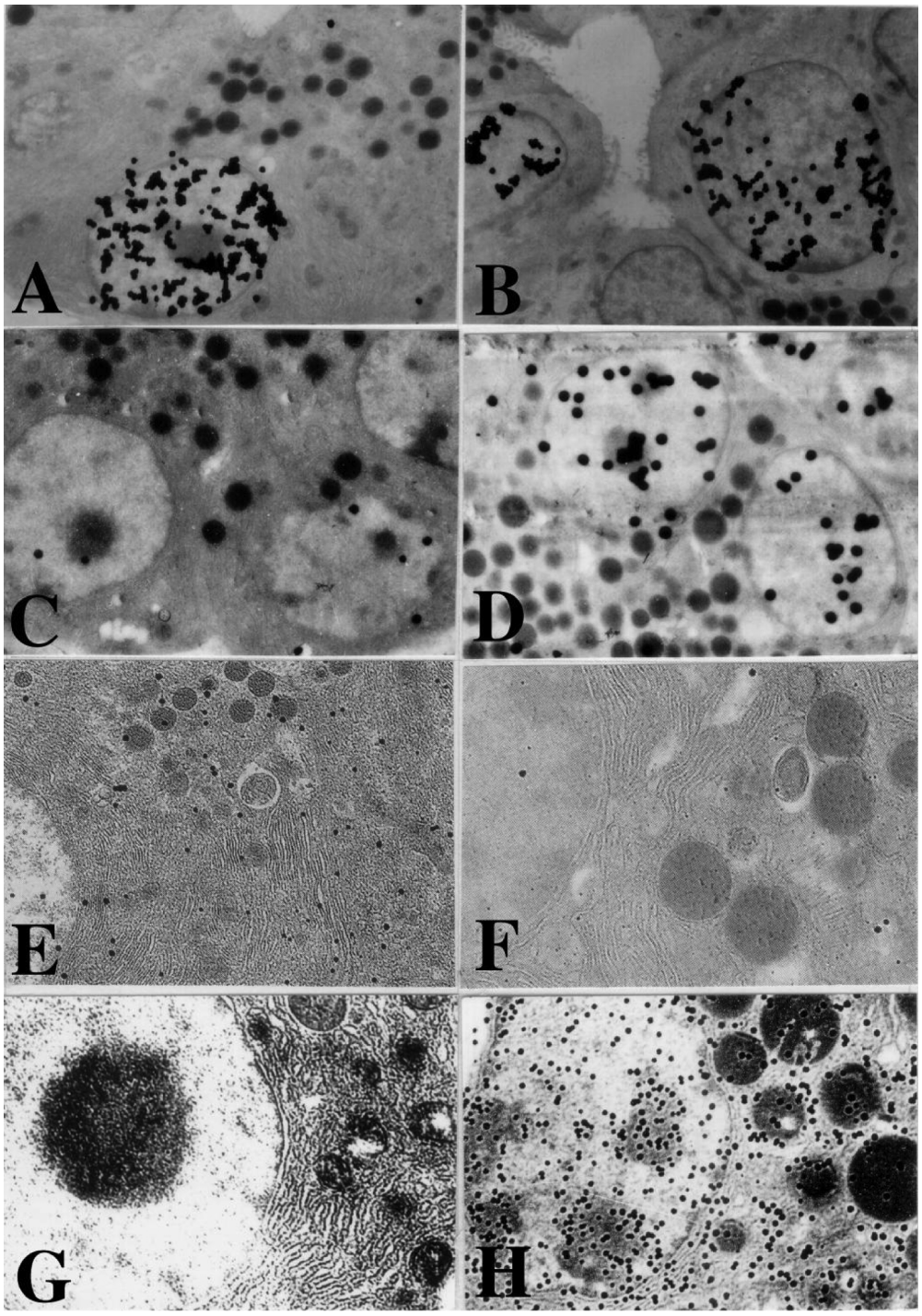

Fig. 6. EM RAG of the pancreas

Fig. 6A. EM RAG of 2 pancreatic acinar cells of a 14 day old mouse labeled with 3H-thymidine, showing DNA synthesis. x10,000
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Fig. 6B. EM RAG of 2 centro-acinar cells of a 14 day old mouse labeled with 3H-thymidine, showing DNA synthesis. x10,000
Fig. 6C. EM RAG of 3 pancreatic acinar cells of a 1 day old mouse labeled with 3H-uridine, showing RNA synthesis. x10,000
Fig. 6D. EM RAG of 3 pancreatic acinar cells of a 14 day old mouse labeled with 3H-uridine, showing RNA synthesis. x10,000
Fig. 6E. EM RAG of a pancreatic acinar cell of a 30 day old mouse labeled with 3H-leucine, showing protein synthesis. x10,000
Fig. 6F. EM RAG of a pancreatic acinar cell of a 12 month old mouse labeled with 3H-leucine, showing protein synthesis. x10,000
Fig. 6G. EM RAG of a pancreatic acinar cell of a 1 day old mouse labeled with 3H-glucosamine, showing glucide synthesis. x10,000
Fig. 6H. EM RAG of a pancreatic acinar cell of a 14 day old mouse labeled with 3H-glucosamine, showing glucide synthesis. x10,000

2.1.6 The DNA synthesis in the pancreas

The pancreas is a large gland, next to the liver in men and animals, among the digestive glands connected to the intestines. It consists of exocrine and endocrine portions and takes the shape of a compound acinous gland. The exocrine portion is composed of ductal epithelial cells, centro-acinar cells, acinar cells and connective tissue cells, while the endocrine portion, the islet of Langerhans, is composed of 3 types of endocrine cells, A, B, C cells and connective tissue cells. Intracellular transport of secretory proteins in the pancreatic exocrine cells were formerly studied by [122] Jamieson and Palade (1967) by EMRAG. We studied the macromolecular synthesis of the aging mouse pancreas at various ages. We first studied the DNA synthesis of mouse pancreas by LM and EMRAG using ^{3}H-thymidine [123,124]. Light and electron microscopic radioautograms of the pancreas revealed that the nuclei of pancreatic acinar cells (Fig.*6A*), centro-acinar cells (Fig.*6B*), ductal epithelial cells, and endocrine cells were labeled with ^{3}H-thymidine. The labeling indices of these cells in 5 groups of litter mate mice, fetal day 15, postnatal day 1, 20, 60 (2 months) and 730 (2 years) were analyzed. The labeling indices of these cells reached the maxima at day 1 after birth and decreased gradually to 2 years. The maximum in the acinar cells proceeded to the ductal and centro-acinar cells, suggesting that the acinar cells completed their development earlier than the ductal and centro-acinar cells [123,124].

3. 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 [25,26,27,29,36,37,125,]. 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. The skeletal system, the muscular system or the circulatory system were not so much studied.

3.1 The RNA Synthesis in the Digestive System

We have mainly studied the digestive system, but not all the digestive organs yet concerning to the RNA synthesis. Study on RNA synthesis was carried out on the small intestines and the liver and the pancreas.

3.1.1 The RNA synthesis in the intestines

We studied the RNA synthesis of the small intestines of mice after feeding or refeeding under the restricted conditions [145]. Five groups of ddY mice, each consisting of 5 individuals, total 25, were injected with ^{3}H-uridine, an RNA precursor, and sacrificed at different time intervals after feeding. The animals of the first group were injected with ^{3}H-uridine at 9 a.m. and fed at 10 a.m. for 30 min. and sacrificed at 11 a.m. 1 hour after the feeding and 2 hours after the injection, the 2nd group was sacrificed at 1 p.m. 3hours after feeding and 4 hours after the injection, the 3rd group at 5 p.m., 7 and 8 hours later, the 4th group at 9 a.m. on the next day 23 and 24hours later, and finally the 5th group at 1. p.m. on the next day 3 hours after refeeding and 28 hours after the injection. Then, the jejunums obtained from each animal were prepared for isolated cell radioautograms according to [146]. The results demonstrated that the grain counts in mononucleate villus cells reached the maximum (20-30 grains per cell) 4 hours after injection and decreased (10-20/cell) after 28 hours, while the counts in mononucleate villus cells only increased gradually from 4 hours (10/cell) to 28 hours (20/cell). In contrast to this, the grain counts of binucleate cells which appeared in villus cells increased parallely to the mononucleate villus cells (10-20/cell). It was concluded that the RNA synthesis in the jejunal epithelial cells was high in the following order: mononucleate crypt cells, binucleate cells and mononucleate villus cells. These results revealed that the feeding or refeeding affected the RNA synthesis of the intestinal epithelial cells [126].

We also studied intracellular localization of mRNA in adult rat hepatocytes localizing over the peroxisomes by means of in situ hybridization technique [127,128,129]. However, its relationship to the aging of animals was not yet studied.

3.1.2 The RNA synthesis in the liver

The RNA synthesis in the liver was studied by ^{3}H-uridine RAG. When the RI-labeled precursor ^{3}H-uridine was administered to experimental animals, or cultured cells were incubated in a medium containing ^{3}H-uridine in vitro and LM RAG was prepared, silver grains first appeared over the chromatin of the nucleus and nucleolus of all the cells within several minutes, then silver grains spread over the cytoplasm within 30 minutes showing messenger RNA and ribosomal RNA [126,131,132,146,147]. We studied quantitative changes of RNA synthesis in the livers of adult mice before and after feeding by incorporations of ^{3}H-uridine. Five groups of ddY mice, each consisting of 5 individuals, total 25, were injected with ^{3}H-uridine and sacrificed at different time intervals. The animals of the first group were injected with ^{3}H-uridine at 9 a.m. and fed at 10 a.m. for 30 min. and sacrificed at 11 a.m. 1 hour after the feeding and 2 hours after the injection, the 2nd group was sacrificed at 1 p.m. 3hours after feeding and 4 hours after the injection, the 3rd group at 5 p.m., 7 and 8 hours later, the 4th group at 9 a.m. on the next day 23 and 24 hours later, and finally the 5th group at 1. p.m. on the next day 3 hours after refeeding and 28 hours after the injection. Then, the livers were taken out from each animal, prepared for isolated cell radioautograms according to [133,134]. The results demonstrated that the grain counts in both mononucleate and binucleate hepatocytes before feeding (15-25 grains per cell)

increased 4 hours after feeding (30-40 grains per cell), reached the maximum in 24hours (40-50 grains per cell), then decreased on the next day (30-40 grains per cell). It was concluded that the RNA synthesis in the binucleate hepatocytes was a little higher than the mononucleate hepatocytes at the same stages and both increased and decreased after feeding. These results revealed that the feeding or refeeding affected the RNA synthesis of the livers [126].

Then, we studied aging changes of ^{3}H-uridine incorporation in the livers and pancreases of aging mice at various ages from prenatal embryos to postnatal aged mice by LM and EMRAG (Ma and Nagata 1990b, Nagata 1999c). When aged mice were injected with ^{3}H-uridine, LM and EM RAG showed that silver grains were localized over the nucleoli, nuclear chromatin (both euchromatin and heterochromatin), mitochondria and rough surfaced endoplasmic reticulum of hepatocytes (Fig.4C) and other types of cells such as sinusoidal endothelial cells, Kupffer's cells, Ito's fat-storing cells (Fig. 4D), ductal epithelial cells, fibroblasts and haematopoietic cells in the livers at various ages. By quantitative analysis, the total number of silver grains in nucleus, nucleolus and cytoplasm of each hepatocyte increased gradually from fetal day 19 to postnatal days, reached the maximum at postnatal day 14 (30%), then decreased to 24 months (5%). The number of silver grains in nucleolus, when classified into two compartments, grains over granular components and those over fibrillar components both increased paralelly after birth, reached the maxima on day 14 (granular 6-7, fibrillar 1-2/per cell), then decreased to 24 months with aging. However, when the ratio (%) of silver grains over euchromatin, heterochromatin of the nuclei and granular components and fibrillar components of the nucleoli are calculated, the ratio remained constant at each aging point.

3.1.2.1 Mitochondrial RNA synthesis in hepatocytes

The intramitochondrial RNA synthesis was first found in the cultured HeLa cells and the cultured liver cells in vitro using EM RAG [135,136]. Then, it was also found in any other cells in either in vitro or vivo [137,138]. Observing light microscopic radioautograms labeled with ^{3}H-uridine, the silver grains were found over both the karyoplasm and cytoplasm of almost all the cells not only at the perinatal stages from embryo day 19 to postnatal day 1, 3, 9, 14, but also at the adult and senescent stages from postnatal month 1 to 2, 6, 12 and 24 [89,115,140,148,149,150]. By electron microscopic observation, silver grains were detected in most mononucleate hepatocytes in respective aging groups localizing 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 from perinatal stage at embryonic day 19, postnatal day 1, 3, 9, 14, to adult and senescent stages at postnatal month 1, 2, 12 and 24. The silver grains were also observed in binucleate hepatocytes at postnatal day 1, 3, 9, 14, month 1, 2, 6, 12 and 24. The localizations of silver grains over the mitochondria were mainly on the mitochondrial matrices but a few over the mitochondrial membranes and cristae when observed by high power magnification.

As the results, it was found that almost all the hepatocytes were labeled with silver grains showing RNA synthesis in their nuclei and mitochondria. Preliminary quantitative analysis on the number of mitochondria in 10 mononucleate hepatocytes whose nuclei were intensely labeled with many silver grains (more than 10 per nucleus) and other 10 mononucleate hepatocytes whose nuclei were not so intensely labeled (number of silver grains less than 9) in each aging group revealed that there was no significant difference between the number of mitochondria, number of labeled mitochondria and the labeling indices in both types of hepatocytes ($P<0.01$). Thus, the number of mitochondria and the labeling indices were

calculated in 10 hepatocytes selected at random in each animal in respective aging stages regardless whether their nuclei were very intensely labeled or not. The results obtained from the number of mitochondria in mononucleate hepatocytes per cellular profile area showed an increase from the prenatal day (mean ± standard deviation 26.2±/cell) to postnatal day 1 to day 14 (38.4-51.7/cell), then to postnatal month 1-2 (53.7-89.2/cell), reaching the maximum, then decreased to year 1-2 (83.7-80.4/cell) and the increase was stochastically significant ($P<0.01$). The results of visual grain counts on the number of mitochondria labeled with silver grains obtained from 10 mononucleate hepatocytes of each animal labeled with ^{3}H-uridine demonstrating RNA synthesis in 10 aging groups at perinatal stages, prenatal embryo day 19, postnatal day 1, 3, 9 and 14, month 1, 6 and year 1 and 2, were counted. 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, respectively. The results showed that the numbers of labeled mitochondria with ^{3}H-uridine showing RNA synthesis increased from prenatal embryo day 19 (3.3/cell) to postnatal month 1 (9.2/cell), reaching the maximum, and then decreased to month 6 (3.5/cell) and again increased to year 1 (4.0/cell) and year 2 (4.3/cell), while the labeling indices increased from prenatal day 19 (12.4%) to postnatal month 1 (16.7%), reaching the maximum, then decreased to year 1 (4.8%) and year 2 (5.3%). Stochastical analysis revealed that the increases and decreases of the number of labeled mitochondria from the perinatal stage to the adult and senescent stage were significant in contrast that the increases and decreases of the labeling indices were not significant ($P<0.01$). As for the binucleate hepatocytes, on the other hand, because the appearances of binucleate hepatocytes were not so many in the embryonic stage, only several binucleate cells (5-8 at least) at respective stages when enough numbers of binucleate cells available from postnatal day 1 to year 2 were analyzed. The results of visual counts on the number of mitochondria labeled with silver grains obtained from several (5 to 8) binucleate hepatocytes labeled with ^{3}H-uridine demonstrating RNA synthesis in 8 aging groups at perinatal stages, postnatal day 1, 9, 14, and month 1, 2, 6, and year 1 and 2, were counted and 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 calculated, respectively. The results showed that the number of labeled mitochondria increased from postnatal day 1 (2.3/cell) to day 9 (5.2/cell) and remained almost constant around 4-5, but the labeling indices increased from postnatal day 1 (2.1%) to postnatal day 9 (13.6%), remained almost constant around 13% (12.5-13.6%) from postnatal day 9 to month 1, then decreased to month 2 (6.1%) to month 6 (3.9%), and slightly increased to year 1 (6.3%) and 2 (5.3%). The increases and decreases of the number of labeled mitochondria and the labeling indices in binucleate hepatocytes were stochastically not significant ($P<0.01$).

4. THE PROTEIN SYNTHESIS

The proteins found in animal cells are composed of various amino-acidsthat 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 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 [21,22,23,25,26,29, 85]. 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.

4.1 The Protein Synthesis in the Digestive System

We studied the protein synthesis of the stomach and the intestines in the digestive tracts as well as the liver and the pancreas among the digestive glands of rats and mice.

4.1.1 Protein synthesis in the stomach

We observed the secretion process in G-cells by EM RAG using ^{3}H-amino acid [151,152, 153](Sato 1978, Sato et al. 1977, Komiyama et al. 1978). When the stomach tissues were taken out from the adult Wistar rats at postnatal month 1 and were labeled with either ^{3}H-glutamic acid or ^{3}H-glycine in vitro at varying time intervals, silver grains in the EM radioautograms appeared first over the Golgi zones, then migrated to secretory granules and were stored in the cytoplasm, suggesting the secretory kinetics. We also studied the mechanism of serum albumin passing through the gastric epithelial cells into the gastric cells by EM RAG [152]. When adult Wistar rat stomach tissues were labeled with ^{132}I-albumin in vitro at varying time intervals, silver grains in the radioautograms appeared over rough endoplasmic reticulum within 3 min, then moved to the Golgi apparatus in 10 min, and on to secretory granules and into the lumen in 30 min, suggesting the pathway of serum albumin absorption from the blood vessels through the gastric mucous epithelial cells into the gastric lumen [153]. These results demonstrated that the stomach cells of adult rats synthesized proteins and secreted. However, aging changes of these protein synthesis between the young and senescent animal were not yet completed.

4.1.2 Protein synthesis in the intestines

We first studied the incorporations of ^{3}H-leucine and ^{3}H-tryptophane in mouse small intestines in connection to the binuclearity before and after feeding [154]. The results showed that the incorporations of both amino acids were greater in binucleate intestinal epithelial columnar cells than mononucleate villus and crypt cells at both before and after feeding. However, the aging changes of these incorporations were not studied.

4.1.3 Protein synthesis in the liver

As for the protein synthesis in the liver, we first studied the incorporations of ^{3}H-leucine and ^{3}H-tryptophane in mouse hepatocytes in connection to the binuclearity before and after feeding [76,154,155]. The results showed that the incorporations of both amino acids were greater in binucleate hepatocytes than mononucleate. When ^{3}H-leucine was injected into several groups of mice at various ages and the liver tissues were processed for LM and EM RAG, silver grains were observed over all cell types of the liver, i.e., hepatocytes (Fig. 4E), sinusoidal endothelial cells (Fig. 4F), ductal epithelial cells, Kupffer's cells, Ito's fat storing cells, fibroblasts and haematopoietic cells. In hepatocytes, number of silver grains in cytoplasm and karyoplasm increased from perinatalanimals to postnatal 1 month young adult animals and decreased with aging to senescence at 24 months. Number of silver grains observed over respective cell organelles, the Golgi apparatus, mitochondria and endoplasmic reticulum, changed with aging, reaching the maxima at 1 month but the ratio

remained constant at each point. When ^{3}H-proline was injected into mice at various ages from prenatal embryos to postnatal senescence and quantitative changes of collagen and protein synthesis in the livers were studied by electron microscopic radioautography [156,157]. The silver grains due to ^{3}H-proline showing collagen synthesis were localized over the nuclei, cytoplasmic matrix, endoplasmic reticulum, the Golgi apparatus, mitochondria and peroxisomes of almost all the cells such as hepatocytes (Fig. 4F), sinusoidal endothelial cells, Kupffer's cells (Fig. 4G), Ito's fat-storing cells, ductal epithelial cells, fibroblasts and haematopoietic cells at various ages. The number of silver grains in the cell bodies and nuclei, cytoplasmic matrix, endoplasmic reticulum, mitochondria, the Golgi apparatus and peroxisomes of hepatocytes gradually increased from embryo, reaching the maxima at postnatal month 1 and 6, and decreased with aging until 24 months. The grain counts of the cell bodies reached the maximum at month 6 and the nuclei at month 2, while that of endoplasmic reticulum at month 6 and mitochondria at month 1. The number of silver grains localized over the extracellular collagen fibrils and matrices was not so many in respective aging groups and did not show any remarkable changes with aging. From the results, it was concluded that ^{3}H-proline was incorporated not only into collagen but also into the structural proteins of hepatocytes and increased and decreased due to aging under normal aging conditions.

4.1.3.1 Mitochondrial protein synthesis in hepatocytes

When the aging mice at various ages from embryo to senescence were injected with ^{3}H-leucine, it was found that almost all the hepatocytes, from embryonic day 19, postnatal day 1, 3, 9, 14, to adult and senescent stages at postnatal month 1, 2, 12 and 24, incorporated silver grains (Fig. 4E). The silver grains were also observed in binucleate hepatocytes at postnatal day 1, 3, 9, 14, month 1, 2, 6, 12 and 24 [90,91,118,119,139,157,158,159, 160,161,162]. The localizations of silver grains observed over the mitochondria were mainly on the mitochondrial matrices but a few over their nuclei, cytoplasmic matrix, endoplasmic reticulum, ribosomes, Golgi apparatus and mitochondria [157,158,160,161,162]. In the mitochondria the silver grains were localized over the mitochondrial membrans and cristae when observed by high power magnification. Preliminary quantitative analysis on the number of mitochondria in 20 mononucleate hepatocytes whose nuclei were intensely labeled with many silver grains (more than 10 per nucleus) and other 20 mononucleate hepatocytes whose nuclei were not so intensely labeled (number of silver grains less than 9) in each aging group revealed that there was no significant difference between the number of mitochondria, number of labeled mitochondria and the labeling indices in both types of hepatocytes ($P<0.01$).

On the other hand, the numbers of mitochondria, the numbers of labeled mitochondria and the labeling indices were calculated in 10 binucleate hepatocytes selected at random in each animal in respective aging stages, regardless whether their nuclei were very intensely labeled or not, except the prenatal stage at embryonic day 19, because no binucleate cell was found at this stage, resulted in no significant difference between them. Thus, the numbers of mitochondria, the numbers of labeled mitochondria and the labeling indices were calculated in 20 hepatocytes selected at random in each animal in respective aging stages regardlesswhether their nuclei were very intensely labeled or not. The results obtained from the total numbers of mitochondria in mononucleate hepatocytes showed an increase from the prenatal day (34.5/cell) to postnatal days 1 (44.6/cell), 3 (45.8/cell), 9 (43.6/cell), 14 (48.5/cell), to postnatal months 1 (51.5/cell), 2 (52.3/cell), reaching the maximum at month 6 (60.7/cell), then decreased to years 1 (54.2/cell) and 2 (51.2/cell). The increase and decrease were stochastically significant ($P<0.01$). The results obtained from visual counting

on the numbers of mitochondria labeled with silver grains from 20 mononucleate hepatocytes of each animal labeled with ^{3}H-leucine demonstrating protein synthesis in 10 aging groups at perinatal stages, prenatal embryo day 19, postnatal day 1, 3, 9 and 14, month 1, 2, 6 and year 1 and 2, were counted. The labeling indices in respective aging stages were calculated from the numbers of labeled mitochondria and the numbers of total mitochondria per cell. The results showed that the numbers of labeled mitochondria with 3H-leucine showing protein synthesis increased from prenatal embryo day 19 (8.3/cell) to postnatal days 1 (9.6/cell), 3 (8.1/cell), 9 (8.9/cell), 14 (9.5/cell), and month 1 (11.2/cell), reaching the maximum, and then decreased to months 2 (9.1/cell), 6 (8.8/cell) to years 1 (6.7/cell) and 2 (2.2/cell), while the labeling indices increased from prenatal day 19 (20.1%) to postnatal days 1 (21.2%), 3 (21.6%), 9 (22.2%), 14 (23.1%), reaching the maximum, then decreased to month 1 (21.7%), 2 (17.4%), 6 (14.6%), and years 1 (12.4%) and 2 (4.4%). Stochastical analysis revealed that the increases and decreases of the numbers of labeled mitochondria as well as the labeling indices from the perinatal stage to the adult and senescent stages were significant ($P<0.01$).

The results obtained from the numbers of mitochondria in binucleate hepatocytes showed an increase from the postnatal days 1 (66.2/cell), to 3 (66.4/cell), 14 (81.8/cell), to postnatal months 1 (89.9/cell), 2 (95.1/cell), and 6 (102.1), reaching the maximum at month 12 (128.0/cell), then decreased to years 2 (93.9/cell). The increase and decrease were stochastically significant ($P<0.01$). The results obtained from visual counting on the numbers of mitochondria labeled with silver grains from 10 binucleate hepatocytes of each animal labeled with ^{3}H-leucine demonstrating protein synthesis in 10 aging groups at postnatal day 1, 3, and 14, month 1, 6 and year 1 and 2, were counted. The labeling indices in respective aging stages were calculated from the numbers of labeled mitochondria and the numbers of total mitochondria per cell which showed that the numbers of labeled mitochondria with ^{3}H-leucine showing protein synthesis increased from postnatal day 1 (7.3/cell) to day 3 (6.8/cell), 14 (10.2/cell), and month 1 (15.0/cell), 2 (15.9/cell), reaching the maximum at month 6 (19.6/cell), then decreased to year 1 (8.3/cell) and 2 (5.1/cell), while the labeling indices increased from postnatal day 1 (11.8%) to 3 (10.2%), 14 (12.5%), month 1 (18.3%) and 2 (18.7%), reaching the maximum at month 6 (19.2%), then decreased to year 1 (6.4%) and 2 (5.5%). Stochastical analysis revealed that the increases and decreases of the numbers of labeled mitochondria as well as the labeling indices from the newborn stage to the adult and senescent stages were significant ($P<0.01$).

4.1.4 The protein synthesis in the pancreas

As for the protein synthesis in the pancreas, ^{3}H-leucine incorporation into endoplasmic reticulum, Golgi apparatus and to secretory granules of pancreatic acinar cells was first demonstrated by Jamieson and Palade [121]. We first studied ^{3}H-glycine incorporation into these cell organelles of mouse pancreatic acinar cells in connection with soluble compounds by EM RAG [89,163,164]. It was demonstrated that soluble ^{3}H-glycine distributed not only in these cell organelles but also in the karyoplasm and cytoplasm diffusely. Then, the quantitative aspects of protein synthesis with regards the aging from fetal day 19, to postnatal day 1, 3, 7, 14 and 1, 2, 5 and 12 months were also clarified [163,165]. The results showed an increase of silver grain counts labeled with 3H-leucine after birth, reaching a peak from postnatal 2 weeks to 1 month (Fig.6E), and decreasing from 2 months to 1 year (Fig. 6F).

On the other hand, we also studied ^{3}H-leucine incorporations into the pancreatic acinar cells of both normal adult rats and experimentally pancreatitis induced rats with either ethionine or

alcohol [165,166]. The results showed that the incorporations as indicated silver grain counts in the pancreatitis rats were less than normal control rats. However, its relation to the aging was not yet studied.

5. 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 [22,23,24,35,57,61,85,167]. 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.

5.1 The Glucide Synthesis in the Digestive System

Among several digestive organs in the digestive tracts and the digestive glands, we studied the glucide synthesis of the stomach and the intestines in the digestive canals as well as the salivary gland, the liver and the pancreas in the digestive glands of aging mice.

5.1.1 The glucide synthesis in the oral cavity

In the oral cavity, we studied the incorporations of ^{3}H-glucosamine in the submandibular glands of 10 groups of litter mice at various ages. The animals from embryonic day 19, postnatal day 1, 3, 7, 14, and 1, 3, 6 months to 1 and 2 years were sacrificed after administration of ^{3}H-glucosamine and the submandibular glands were processed for LM and EM RAG [85,168]. The results showed that the silver grains appeared over the endoplasmic reticulum, Golgi apparatus and the secretory granules of the acinar cells, demonstrating the glycoprotein synthesis in these cells. Grain counting revealed that the counts increased from the fetal stage at embryonic day 19 to postnatal day 1 to 3, 7,14, reaching the peak at day 14, then decreased to month 1, 3, 6, to year 1 and 2, showing the aging changes, inverse proportion to DNA synthesis of these cells.

The sulfate uptake and accumulation in sulfomucin in several digestive organs of mice were also studied by light microscopic radioautography [169,170]. Two litters of normal ddY mice

30 days after birth, each consisting of 3 animals, were studied. One litter of animals was sacrificed at 30 min after the intraperitoneal injections with phosphate buffered $Na_2{}^{35}SO_4$, and the other litter animals were sacrificed at 12hr after the injections. Then the submandibular glands and the sublingual glands were taken out, fixed, embedded in epoxy resin, sectioned, radioautographed and analyzed by light microscopy. As the results, many silver grains were observed on serous cells of the salivary glands at 30 min and 12hr after the injections (10-20/cell). The numbers of silver grains at 30 min were less than those at 12 hr. From the results, it was concluded that glycoprotein synthesis was demonstrated in both the submandibular and sublingual glands by radiosulfate incorporation. In the salivary glands the silver grains were more observed in serous cells than mucous cells at 30 min, while in mucous cells more at 12hr than 30 min after the injection. These results show the time difference of glycoprotein synthesis in the two salivary glands, showing inverse proportion to DNA synthesis of these cells [85,168].

5.1.2 The glucide synthesis of the stomach

When incorporation of radiosulfate into sulfated complex carbohydrate in rat stomach was studied by labeling with $^{35}SO_4$ in vivo, silver grains appeared over the glandular cells of the pyloric gland but not those of the fundic gland, demonstrating the mucous synthesis in the former glands [169,171]. The radiosulfate uptake and accumulation in the stomach of mouse were also studied by light microscopic radioautography [170]. Two litters of normal ddY mice 30 days after birth, each consisting of 3 animals, were studied. One litter animals were sacrificed at 30 min after the intraperitoneal injections with phosphate buffered $Na_2{}^{35}SO_4$, and the other litter animals were sacrificed 12hr after the injections. Then the antrum and the fundus tissues of the stomachs were taken out. The tissues were fixed, dehydrated, embedded in epoxy resin, sectioned, radioautographed and analyzed. As the results, many silver grains were observed on the mucosa and submucosa of the stomach at 30 min after the injection. Then at 12hr after the injection silver grains were observed on some of the fundic glands. The numbers of silver grains observed in the stomach especially over the pyloric glands at 30 min (a few per cell) were less than those (several per cell) at 12 hr. The results showed the time difference of glycoprotein synthesis in the stomach, showing inverse proportion to DNA synthesis [138,169].

5.1.3 The glucide synthesis in the intestines

We also studied the aging changes of glucide synthesis by ^{3}H-glucosamine uptake in the small intestines of mouse (Morita 1993), and found that the silver grains in the ileum columnar epithelial cells were mainly localized over the brush borders and the Golgi regions in these cells (Fig. 1F). The grain counting revealed that the numbers of silver grains over the brush borders and cytoplasm of the columnar epithelial cells increased in the villi (10-15/cell) than in the crypts (1-2/cell) from 6 months up to 2 years due to aging. The grain counting in other cell types also revealed that the number of silver grains in goblet cells, basal granulate cells, Paneth cells increased by aging, but did not in the undifferentiated cells.

The glycoprotein synthesis in goblet cells as well as in absorptive epithelial cells was also studied using $^{35}SO_4$ incorporation in the duodenums, the jejunums and the colons of adult mice at varying time intervals at 30, 60, and 180 min after the administration [169,171,172]. Silver grains were localized over the columnar absorptive cells and the goblet cells, especially over the Golgi regions and mucous granules of the goblet cells. By EM RAG the intracellular localization of silver grains in goblet cells was clearly shown in the Golgi

apparatus. The results from grain counting revealed that the average grain counts were different in the upper and deeper regions of the crypts in the 4 portions and it was shown that silver grains over goblet cells in the lower region of the crypt transferred rapidly from 30 min to 180 min, while they transferred slowly in goblet cells in the upper region of the colonic crypt, leading to the conclusion that the rates of transport and secretion of mucous products of the goblet cells at these two levels in the crypts were different. By EM RAG silver grains first appeared over the Golgi zone at 30 min. and then moved to the secretory granules at 60 and 180 min. The incorporation of $Na_2{}^{35}SO_4$ into sulfated complex carbohydrate was investigated in the mouse small and large intestines by LM and EM RAG as well as in the submandibular glands and the stomachs. Quantitative differences have been observed in the relative uptake of radiosulfate in the various labeled cells of each organ. Incorporation by the colon in goblet cells exceeded that elsewhere in the deep goblet cells of the colonic crypts migration of label progressed during the time tested from the supranuclear Golgi region to the deep position of the goblet and then extended throughout the mucosubstance in the goblet in the superficial goblet cells of the colon. The radioautographic and cytochemical staining differences between secretory cells in the deeper region compared with the upper region of the colonic crypts are considered to reflect differences in the rate of transport of secretory products in the theca and the rate of secretion at the low levels in the crypt (Figs. 1G,1H). These results showed the time differences of glycoprotein synthesis in respective organs. The sulfate uptake and accumulation in several mouse digestive organs were also studied by LM RAG. Two litters of normal ddY mice 30 days after birth, each consisting of 3 animals, were studied. One litter of animals was sacrificed 30 min after the intraperitoneal injections with phosphate buffered $Na_2{}^{35}SO_4$, and the other litter animals were sacrificed 12hr after the injections. Then several digestive organs, the parotid gland, the submandibular gland, the sublingual gland, antrum and fundus of the stomach, the duodenum, the jejunum, the ileum, the caecum, the ascending colon and the descending colon were taken out and radioautographed. As the results, many silver grains were observed on villous cells and crypt cells of the small intestines and whole mucosa of the large intestines at 30 min after the injection. Then at 12hr after the injection silver grains were observed on mucigen granules of goblet cells in the small intestines and the large intestines. The numbers of silver grains observed in respective organs at 30 min were less than those at 12hr. From the results, it was concluded that the time difference of the glycoprotein synthesis was demonstrated in several digestive organs by radiosulfate incorporation, in reverse proportion to DNA synthesis. The total S contents in colonic goblet cells in upper and deeper regions of colonic crypts in aging mice were also analyzed by X-ray microanalysis [173,174]. The results accorded well with the results from RAG [138] showing increase and decrease of mucosubstances in these cells due to development and aging to senescence.

5.1.4 The glucide synthesis in the liver

We first studied ^{3}H-glucose incorporation into glycogen in the livers of adult mice, in connection to soluble compounds [53,175,177]. Soluble ^{3}H-glucose, which was demonstrated by cryo-fixation (at -196ºC) in combination with dry-mounting radioautography, was localized over the nuclei, nucleoli, all the cell organelles and cytoplasmic ground substance of all the hepatocytes diffusely. On the other hand, by conventional chemical fixation and wet-mounting radioautography, silver grains were localized only over glycogen granules, endoplasmic reticulum and Golgi apparatus showing glycogen synthesis. However, the relationship of glycogen synthesis to aging has not yet been fully clarified.

5.1.5 The glucide synthesis in the pancreas

Concerning the glucide synthesis of the pancreas, we first studied the incorporation of ^{3}H-glucose into the pancreatic acinar cells of mouse in connection with soluble compounds by EM RAG [53]. It was demonstrated that soluble ^{3}H-glucose distributed not only in such cell organelles as endoplasmic reticulum, Golgi apparatus, mitochondria but also in the karyoplasm and cytoplasm diffusely. Then, the incorporation of ^{3}H-glucosamine into the pancreases of aging mice at various ages was studied by LM and EM RAG [178,179]. When perinatal baby mice received ^{3}H-glucosamine injections and the pancreatic tissues were radioautographed, silver grains were observed over exocrine and endocrine pancreatic cells. However, the number of silver grains was not so many (Fig. 6G). When juvenile mice at the age of 14 days after birth were examined, many silver grains appeared over the exocrine pancreatic acinar cells (Fig. 6H). Less silver grains were observed over endocrine pancreatic cells and ductal epithelial cells. The grains in the exocrine pancreatic acinar cells were localized over the nucleus, endoplasmic reticulum, Golgi apparatus and secretory granules, demonstrating glycoprotein synthesis. Adult mice at the ages of postnatal 1 month, 6 month or senile mice at the ages of 12 months or 24 months showed very few silver grains on radioautograms. Thus, the glucide synthesis in the pancreatic acinar cells of mice revealed quantitative changes, increase and decrease of ^{3}H-glucosamine incorporation with aging [22,33,24,179,180,181].

6. THE LIPIDS 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 [14,22,23,24,25,29,125,179,180,181]. 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.

6.1 The Lipids Synthesis in the Digestive System

We studied only the livers and the pancreases of aging mice at various ages but only demonstrating soluble compounds by means of cryo-fixation and dry-mounting radioautography.

6.1.1 The lipids synthesis in the liver

We observed lipid synthesis in the liver using ^{3}H-glycerol in connection to soluble compounds [53,180,181,175,176]. When adult mice were injected with ^{3}H-glycerol and the livers were taken out, cryo-fixed in liquid nitrogen at -196ºC, then freeze-substituted,

embedded in epoxy resin, dry-sectioned, and prepared for dry-mounting radioautography, many silver grains appeared over the nuclei and cytoplasm of hepatocytes diffusely. However, when the same liver tissues were fixed chemically in buffered glutaraldehyde and osmium tetroxide at 4ºC, dehydrated, embedded, wet-sectioned and radioautographed by conventional wet-mounting procedures, very few silver grains were observed only over the endoplasmic reticulum and the lipid droplets, which demonstrated insoluble macromolecular lipid synthesis accumulating into the lipid droplets. However, the aging change of the lipid synthesis in the liver has not yet been fully clarified.

6.1.2 The lipid synthesis in the pancreas

In order to demonstrate the lipids synthesis in the pancreas, several litters of ddY mice aged fetal day 19, postnatal day 1, 3, 7, 14, and 1, 2, 6 up to 12 months, were injected with ^{3}H-glycerol and the pancreas tissues were prepared for LM and EM RAG. The silver grains were observed in both exocrine and endocrine cells of respective ages [38,171,182,183]. In perinatal animals from fetal day 19 to postnatal 1, 3, and 7 days, cell organelles were not well developed in exocrine and endocrine cells and number of silver grains was very few. In 14 day old juvenile animals, cell organelles such as endoplasmic reticulum, Golgi apparatus, mitochondria and secretory granules were well developed and many silver grains were observed over these organelles and nuclei in both exocrine and endocrine cells. The number of silver grains was more in exocrine cells than endocrine cells. In 1, 2, 6 month old adult animals, number of silver grains remained constant. In 12 month old senescent animals, silver grains were fewer than younger animals. It was demonstrated that the number of silver grains expressed the quantity of lipids synthesis, which increased from perinatal atages to adult and senescent stages and finally decreased to senescence.

7. 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 [184], neurotransmitters and inhibitors such as ^{14}C-bupranolol, a beta-blocking agent [185] or ^{3}H-befunolol [186,187], vitamins, drugs such as synthetic anti-allergic agent ^{3}H-tranilast [188,189,190,191,192], hypolipidemic agent bezafibrate [193,194,195], calmodulin antagonist [196,197] or anti-hypertensive agent ^{3}H-benidipine hydrochloride [198], toxins, inorganic substances such as mercury [176,199] and others such as laser beam irradiation. The details are referred to the previous publication on the radioautographology [138]. However, their relationships to the cell aging were not studied.

8. CONCLUSION

From the results obtained at present, it was concluded that almost all the cells in various organs of all the organ systems of experimental animals at various ages from prenatal to postnatal development and senescence during the aging of cells and individual animals demonstrated to incorporate various macromolecular precursors such as ^{3}H-thymidine, ^{3}H-uridine, ^{3}H-leucine, ^{3}H-glucose or ^{3}H-glucosamine, ^{3}H-glycerol and others localizing in the nuclei, cytoplasmic cell organelles showing radoautographic silver grains due to DNA, RNA, proteins, glucides, lipids and others those which the cells synthesized during the cell aging. Quantitative analysis carried out on the numbers of silver grains produced by radioautography in respective cell organelles demonstrated quantitative changes, increases

and decreases, of these macromolecular synthesis in connection to cell aging of respective organs.

In general, DNA synthesis with ^{3}H-thymidine incorporations in most organs showed maxima at perinatal stages and gradually decreased due to aging. To the contrary, the other synthesis such as RNA, proteins, glucides and lipids increased due to aging and did not remarkably decrease until senescence. Anyway, these results indicated that macromolecular synthetic activities of respective compounds in various cells were affected from the aging of the individual animals.

Thus, the results obtained from the various cells of various organs should form a part of special radioautographology that I had formerly proposed [85,200], i.e., application of radioautography to the aging of cells, as well as a part of special cytochemistry [201,202], as was formerly reviewed. We expect that such special radioautographology and special cytochemistry should be further developed in all the organs in the future.

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COMPETING INTERESTS

Author has declared that no competing interests exist.

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