Light Microscopic Radioautographic Study on Radiosulfate Incorporation into the Tracheal Cartilage of Aging Mice

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The aging changes of the incorporations of radiosulfate ($^{35}\text{SO}_4$) into the tracheal cartilage were studied in several litters of mice at various ages, from embryos to postnatal 1 year by light microscopic radioautography. The results show that silver grains indicating the incorporations of radiosulfate were found over the cartilage matrices and the cartilage capsules in the hyaline cartilages of the tracheae of perinatal mice. The grain density was at the maximum at fetal day 19, then decreased from fetal day 19 to postnatal day 1, 3, 7, 14 and 30. The silver

grains in the perinatal animals aged at postnatal day 1 and 3, moved from the internal layer to the external layer of the cartilage and from the interterritorial matrix to the territorial matrix and the cartilage capsule. No silver grains were found in the animals aged from 1 to 12 months. From these results, it is concluded that the radiosulfate was incorporated into sulfated complex carbohydrate in the cartilage matrices and the pattern of incorporation in the tracheae decreased with the aging of animals.

Key words: Light microscopic radioautography, Sulfated complex carbohydrate, Mouse trachea, Hyaline cartilage, Aging

I. Introduction

The aging changes of macromolecular synthesis in some organs of mice have been investigated by means of light and electron microscopic radioautography in our laboratory [40–54]. The aging changes of DNA synthesis were demonstrated by ^3H -thymidine incorporations into the skeletal muscles [10], the esophagus [4], the liver [28, 29], the pancreas [52], the intestines [14–16, 36], the lung [61, 62, 64], the trachea [65], the adrenal gland [12, 13], the kidney [9], the testis [5–7], the ovary and uterus [23, 24, 70], the brain [2], and the retina [5, 20, 21] of mice; RNA synthesis was demonstrated by ^3H -uridine incorporations into the liver [30], the pancreas [50], the lung [61], the testis [6], the ovary and uterus [24, 69], the adrenal gland [26, 27], and the retina [22] of mice; the protein synthesis was demonstrated by ^3H -amino acid incorporations such as ^3H -leucine in the liver [32], the pancreas [53], the lung [63], the spleen [52], the testis [6], the uterus [69], the retina [67] and the cornea [3], or ^3H -proline in the liver [31] of mice. Among the macromolecular synthesis, however, radiosulfate incorporations into glycoproteins

have not been studied so much. Only a few papers are available dealing with the incorporations into the digestive organs which secrete mucus such as the salivary glands, stomachs and intestines of adult mice [51, 55] or into the glycoproteins in the ovarian follicular epithelia of mature female mice [25]. Sulfate is supposed to play some important roles as precursors for the glycoproteins and glycosaminoglycans in several organs of the digestive system [33]. It should be interesting to compare the incorporation differences among several organs. However, no literature is available dealing with the radiosulfate incorporations into the respiratory organs during the aging of animals. As a part of the serial studies on the qualitative and quantitative changes of macromolecular synthesis in various organs of aging mice in our laboratory, the incorporation of radiosulfate into the tracheal cartilage was carried out in several litters of ddY strain mice. The preliminary report was presented at the 40th Memorial Scientific Meeting of the Japan Society of Histochemistry and Cytochemistry in Kyoto, December 6–7, 1999 [48]. This paper deals with the contents in detail.

II. Materials and Methods

The animals

Nine groups of ddY strain mice of both sexes, each

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consisting of 3 litter individuals, from fetal day 19, to postnatal 1, 3, 7 and 14 days, 1, 2, 6, and 12 months were used. The embryonic age was based on observation of the vaginal plug of the female mice (vaginal plug=day 0). They were housed under conventional conditions, fed with normal mice chow (Clea CE2, Tokyo, Japan) with access to water *ad libitum* in our laboratory.

Radioautography

The animals were injected with radiosulfate saline solution ($\text{Na}_2^{35}\text{SO}_4$, 3700 kBq/g body weight, purchased from the Radiochemical Center, Amersham, England, UK, specific activity 1.11 TBq/mM) intraperitoneally at 9 a.m. in the morning to exclude the diurnal variation, sacrificed at 10 a.m. by decapitation. In case of embryos, radiosulfate was injected intraperitoneally to the pregnant mouse and the embryos were taken out after the mother was sacrificed. The tracheae were separated from the lungs and cut into tracheal rings in Hanks' solution (Nissui, Tokyo, Japan), fixed in phosphate buffered 10% formalin, rapidly frozen at -78°C in dry ice and acetone mixture, cryo-sectioned in a cryostat (DAMON Minotome, IEC Co., Needham Heights, USA) at -40°C , picked up onto glass slides, air-dried, and radioautographed with Konica NR-M2 radioautographic emulsion (Konica, Tokyo, Japan) by a dipping procedure [37, 38], exposed for 10 weeks at 4°C , developed with SDX-1 developer at 20°C for 5 min, fixed in an acid hardening fixer (sodium thiosulfate solution) for 10 min, stained with H-E and air-dried. Radioautograms were observed and photographed with an Olympus Vanox AHB-LB light microscope (Olympus Opt. Co., Tokyo, Japan).

Quantitative analysis

In order to compare the relative amount of radiosulfate incorporations into the tracheal cartilage in each animal in respective groups, the number of silver grains per unit area (1 mm^2) in the central portions of the hyaline cartilage in 10 sections in each animal from respective groups picked up at random was counted by means of a computer assisted image analyzing system, Luzex III (Nireco Co., Tokyo, Japan) for quantitative analysis [39]. From all the data thus obtained the averages and standard deviations in respective age groups were computed with a personal computer Macintosh LC475 (Apple Computer Japan, Tokyo, Japan). The data were statistically analyzed using variance and Student's *t*-test. The differences among age groups were considered to be significant when *P* value was ≤ 0.01 .

III. Results

Qualitative observations

The results showed that silver grains indicating the incorporation of radiosulfate were mainly found over the cartilage matrices both in the interterritorium and the territorium especially over the cartilage capsules surrounding

the chondrocytes in the hyaline cartilages of the tracheae of perinatal mice. The number of silver grains appeared most densely distributed at fetal day 19. The silver grains at fetal day 19 accumulated over the cartilage matrices excluding the chondrocytes (Fig. 1). Only a small number of silver grains can be seen over the cytoplasm of the chondrocytes. The silver grains in the perinatal animals aged at postnatal day 1 accumulated over the interterritorial and territorial matrices in the outer layer of the cartilage but the number of grains decreased in the inner layer (Fig. 2). At postnatal day 3, the silver grains disappeared from the most interterritorial and the territorial matrices in both the internal layer and the external layer of the cartilage except for several groups of territorial matrices, surrounding several territorial capsules including several chondrocytes, where much more intense accumulation of silver grains was disseminatedly noticed than other areas (Fig. 3). In the newborn animals aged at postnatal day 7, intense incorporations were observed disseminating over several groups of cartilage capsules in the external layer but not in the internal layer (Fig. 4). In the juvenile animals at postnatal day 14, less intense silver grains were observed over some groups of several territorial matrices disseminating in the outer layer of the cartilage (Fig. 5). However, no silver grains were found in animals aged from 1, 2, 6 to 12 months. In the columnar epithelial cells of the tracheae, on the other hand, only a few silver grains were noticed over the secretory granules of goblet cells in the respective groups.

Quantitative analysis

The results obtained by grain counting using an image analyzing system are depicted in Fig. 6. The number of silver grains, as expressed by grain density per unit area (1 square mm) was maximum at fetal day 19. The grain density then decreased from fetal day 19 to postnatal day 1, 3, 7 and 14 and reached 0 from 1 month to 1 year (Fig. 6). The quantitative data showed that the incorporation of radiosulfate into the cartilage matrix was the highest at the perinatal stage and decreased gradually from postnatal day 3 to postnatal day 14, then stopped at adult stage from 1 month to 1 year with the aging of animals. The decrease of the grain density from fetal day 19 to postnatal day 14 was stochastically significant at $P \leq 0.01$.

IV. Discussion

We have studied the macromolecular synthesis such as DNA, RNA, glycoproteins and proteins in various organ systems of aging mice [40–56]. In the present study, we have first studied the incorporations of radiosulfate in the tracheal tissues in aging mice and found that the radiosulfate was incorporated only into the tracheal cartilage matrix and the grain density per unit area was the highest at fetal day 19. The grain density then decreased from fetal day 19 to postnatal day 1, 3, 7 and 14 and reached 0 from 1 month to 1 year. There is a possibility that a

Figs. 1-5

maximum may occur during the fetal period from day 1 to 19. However, due to the difficulty in taking tracheal cartilage samples from respective fetal days, only day 19 immediately before the birth was studied at present. The number of silver grains localized over the chondrocytes was less than the cartilage matrix at respective aging stages. The reason for the accumulation of silver grains over the cartilage matrix may be because the fixation of tissues was carried out 1 hr after the injection of the radiosulfate when the label moved from the chondrocytes, which incorporated the radiosulfate into their cell bodies at early time intervals, later to the cartilage matrix. The radiosulfate incorporation into the cartilage was previously observed by the uptake of $^{35}\text{SO}_4$ into the chondrocytes at 15 or 30 min after the injection and the label migrated from the chondrocytes to the amorphous matrix at later time intervals [8]. The radiosulfate incorporation into the amorphous cartilage matrix was supposed to form the constituents of the ground substance, the complex carbohydrate or glycoprotein, a copolymer of chondroitin-4-sulfate and chondroitin-6-sulfate, which is responsible for the basophilia and the metachromasia of the cartilage matrix [1, 60]. The results obtained at present demonstrating more intensive incorporations of radiosulfate over the territorial matrices or cartilage capsules than the interterritorial matrices at respective perinatal stages suggest that the concentration of radiosulfate in the territorial matrices was more than the interterritorial matrices, in accordance with the staining characteristics of the territorial matrices [1, 60]. The time difference in incorporations of radiosulfate into the territorial and interterritorial

Fig. 1. Light microscopic radioautogram of the hyaline cartilage of the trachea of a mouse embryo at fetal day 19. Note that the numerous silver grains showing the incorporations of the radiosulfate accumulate over the interterritorial and territorial matrices but not so much over the chondrocytes. Magnifications in Figs. 1-5 are the same. Scale bar = 50 μm .

Fig. 2. Light microscopic radioautogram of the hyaline cartilage of the trachea of a newborn mouse at postnatal day 1. Note that the number of silver grains accumulating over the interterritorial and territorial matrices decreased from the internal layer of the cartilage.

Fig. 3. Light microscopic radioautogram of the hyaline cartilage of the trachea of a newborn mouse at postnatal day 3. Note that the number of silver grains accumulating over the interterritorial and territorial matrices decreased from the internal layer of the cartilage to the external layer. Intense incorporation of the radiosulfate is observed disseminatedly over several groups of cartilage capsules.

Fig. 4. Light microscopic radioautogram of the hyaline cartilage of the trachea of a newborn mouse at postnatal day 7. Note that the number of silver grains accumulating over the interterritorial and territorial matrices decreased from the internal layer of the cartilage to the external layer. Intense incorporations of the radiosulfate are observed disseminating over several groups of cartilage capsules in the external layer.

Fig. 5. Light microscopic radioautogram of the hyaline cartilage of the trachea of a juvenile mouse at postnatal day 14. Note that the silver grains disappeared from the internal layer of the cartilage. Less intense incorporations of the radiosulfate are observed disseminating over several groups of cartilage capsules in the external layer.

Fig. 6. Transitional curve of grain counts per unit area (1 mm^2), showing radiosulfate incorporations into mouse tracheal cartilage with aging. F19d, fetal day 19; P1d, postnatal day 1; P3d, postnatal day 3; P7d, postnatal day 7; P14d, postnatal day 14; P1M, postnatal month 1; P2M, postnatal month 2; P6M, postnatal month 6; P1Y, postnatal year 1.

matrices between the inner and outer layers at respective aging stages may account for the time difference in the differentiation and function of chondrocytes in these two layers. It is supposed that the chondrocytes in the inner layer of the cartilage become active and differentiated earlier than those in the outer layers and the differentiation proceeds from the inner to the outer layers according to the aging of animals. It is also supposed that the cartilage matrices need to incorporate some sugars and amino acids to synthesize macromolecules of glycoproteins consisting of sugars and proteins. To verify this point, further experiments demonstrating the incorporations of sugar precursors such as ^3H -glucosamine or amino acids such as ^3H -leucine into the cartilage matrices should be necessary like the incorporations of such precursors into the corneal stroma of mouse which were already carried out in our laboratory [49, 54].

Our previous radioautographic observations on the trachea demonstrated that the DNA synthesis in the ciliated epithelial cells was very low but the synthesis in the nonciliated and basal epithelial cells was maximum at embryonic day 18 and decreased from postnatal day 3 onwards keeping a low labeling index around 0.2% from 2 to 12 months [65]. It was also previously suggested that the cell divisions of basal cells give rise to other cell types in normal development, in maintenance of the adult epithelium, and in regeneration following injury, and further to neoplastic cells in tumorigenesis [34]. The basal cells are often referred to as reserve cells [34] or progenitor cells in the regenerating tracheal epithelium [11, 17–19, 57–59]. However, the labeling index of chondrocytes was different from the epithelial cells [65]. It was the highest at embryonic day 18, then gradually decreased from postnatal day 3 on reaching 0 at 2 months until 2 years [65]. The chondrocytes lost the ability to synthesize DNA at 2 months after birth. The results are in good agreement with those of other researchers. Weiss *et al.* [68] found

that the DNA concentration of hyaline cartilage in mice decreased significantly by 1 month of age as compared to newborns, and reached a plateau at the age of 3 months. Our previous study [65] clearly indicated that the chondrocytes could not synthesize DNA and the growth and maturation activity of the chondrocytes attained their completion at 2 months after birth. The decrease in the DNA synthesis that implies a reduced tissue cellularity was substantiated by morphological findings indicating a dramatic decrease in cell density during the first postnatal month. The results obtained at present, demonstrating that the grain density per unit area incorporating radiosulfate was maximum at fetal day 19 then decreased to postnatal day 1, 3, 7 and 14 and reached 0 from 1 month to 1 year, correspond to the chondrocyte proliferation and cartilage growth obtained previously [3].

On the other hand, the mucosubstance synthesis with radiosulfate, $^{35}\text{SO}_4$, was studied in the ovaries of adult mice during the estrus cycle in our laboratory [25]. Four groups of female ddY mice, aged 8–10 weeks, were divided into 4 groups, diestrus, proestrus, estrus and metestrus according to the vaginal smears. The ovaries were taken out, labeled with $^{35}\text{SO}_4$ *in vitro* and radioautographed. In all the animals, silver grains were localized over the granulosa and theca cells. Almost all compartments of the ovary were labeled. The grain counts per cell changed according to the cell cycle. From the results, it was concluded that all the cells of the ovary incorporated mucosubstances throughout the estrus cycle [25].

In the adult mice at postnatal 4 weeks, the stomach, small and large intestines, the duodenum, the jejunum, the caecum and the distal colon, were studied by light microscopic radioautography at 30, 60 and 180 min intervals after injection of radiosulfate [47]. From the results, quantitative differences were 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 deeper goblet cells of the colonic crypts, label migration progressed slowly during the time tested for 180 min but it extended quickly in the goblet in the superficial goblet cells in the crypts [55]. We have also studied 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 of the 2 groups of adult mice, 30 days after birth, 30 min and 12 hr after the radiosulfate injection, respectively [55]. These results show that silver grains were observed in those digestive organs demonstrating glycoprotein synthesis but a time difference in the respective organs was noticed. It is also noteworthy that the incorporations of radiosulfate in the goblet cells of tracheal epithelial cells in the respective aging groups in the present study were less than the cartilage matrices as well as those goblet cells in the intestines in the previous studies. On the other hand, the incorporation of ^3H -glucosamine into the small intestines of aging mice increased after birth and reached the maxima at postnatal 6 months to 2 years [35].

From these results, it is supposed that the glycoproteins synthesized in the digestive organs are secreted and metabolized after the development of these organs. In contrast to these organs, however, the glycoproteins in the tracheal cartilage, which became the constituents of the cartilage matrix, did not show any incorporation of radiosulfate after the cartilage was developed at postnatal 1 month to 1 year. The difference between the incorporation patterns of radiosulfate into digestive and respiratory organs should be due to the organ specificity. These data are an addition to a part of special radioautographology as was recently reviewed by the present author [46, 47]. We expect that such data should be accumulated to form a part of special radioautographology, developing this novel science in all the organ systems in men and animals in the future.

V. Conclusion

From the results obtained, it is concluded that the radiosulfate was incorporated into sulfated complex carbohydrate in the cartilage matrix in the tracheae of perinatal mice and the patterns of incorporation into the tracheal cartilage matrix decreased from the perinatal stage to the adult and senescent stage from postnatal 1 month to 1 year corresponding to the aging of animals.

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