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Ultrastructural Changes in Aging Rat Tracheal Gland

Shigeo KASUGA

Central Research Laboratory, Wakunaga Pharmaceutical Co., Ltd., 1624 Shimokotachi Koda-cho, Takata-gun, Hiroshima 729-64, Japan

ABSTRACT

Ultrastructural differences in the tracheal gland between young and aged rats were studied. Marked differences were observed in the serous cells between the young (8-17 week-old) and the aged (20-30 month-old) subjects. The secretory granules of the serous cells of the young subjects were homogeneously electron dense, whilst those of the aged subjects had a bizonal appearance. These bizonal granules contained abundant mucous material. The *trans* Golgi network of the aged rat serous cells was more developed and formed a fine meshwork in comparison with that of the young subjects. In the aged subjects, findings indicate alterations not only in the function of the Golgi apparatus including formation of secretory granules and addition of terminal sugars to the secretory protein but also the histochemical and ultrastructural properties of the secretory material. The changes in the Golgi apparatus are possibly responsible for depression of the tracheal host defense mechanism and might explain why various airway diseases increase with aging.

Key words: *Aging, Tracheal gland, Golgi apparatus*

The tracheal gland is composed of serous and mucous cells and secretes respiratory tract fluid. This plays an important role in mucociliary clearance together with ciliary beat. The serous cells produce local defensive substances, such as lysozyme, and play a direct role in the tracheal defense mechanisms[2,19]. It is well known that mucociliary clearance is depressed in various respiratory diseases[11,16,20]. However, there are few reports which support the view that an impared mucociliary clearance results in infection of the respiratory tract. It is also known that various airway diseases increase with aging suggesting that mucociliary clearance deteriorates with aging.

Yajin et al[21] have demonstrated that the number of tracheal gland cells decreases in the aged rats and that the histochemical property of their mucosubstances is altered. This might explain why various airway diseases increase with aging.

In the present study, the ultrastructural changes in the rat tracheal gland cells due to aging were observed by means of transmission and scanning electron microscopies (TEM and SEM). In addition, the enzyme cytochemical examination of the Golgi apparatus was achieved.

MATERIALS AND METHODS

Animals: Twenty male Sprague-Dawley rats were used. All rats were specific pathogen free (SPF) animals and purchased at 8 weeks old. They were raised in a designated animal room maintained in a semi-clean condition. The ages of the young group were 8 to 17 weeks old (10 animals) and those of the older group were 20 to 30 months old (10 animals).

Preparation method for TEM specimens: The rats were anesthetized with Nembutal (sodium pentobarbital) and perfused through the left ventricle with 1/2 strengthened Karnovsky fixative[4] containing 2.5% glutaraldehyde and 2% paraformaldehyde in 0.05M sodium cacodylate buffer, pH 7.3, with 0.05% $CaCl_2$. After perfusion, segments of the trachea extending from the cricoid cartilage to the tracheal bifurcation were removed. The tracheas were then divided into right and left segments along the rostro-caudal axis and placed in fresh fixative for 2 hr at 4°C. They were rinsed in the same buffer and trimmed into strips, each containing two cartilage rings. The tissue strips were rinsed overnight in the same buffer and then postfixed for 2 hr at 4°C in 1% OsO_4 in the same buffer. After a brief rinse, the tissues were dehydrated in ethanol and embedded in Epon.

Enzyme cytochemistry: For enzyme cytochemistry, the tracheas were removed in the same manner as mentioned above after perfusion with 1/4 strengthened Karnovsky fixative. The tracheas were opened by cutting the posterior membrane along the rostro-caudal axis, and then placed in 0.05M sodium cacodylate buffer,pH 7.3, containing 7% sucrose (sucrose buffer). The specimens were divided into transverse segments, each consisting of two cartilage rings. These were rinsed several times in sucrose buffer and stored overnight at 4°C. The tissue segments were embedded in the embedding medium for freezing (O.C.T. compound; Ames Division Miles Lab. Inc.), and frozen using liquid nitrogen. Frozen tissues were cut into 20 μm

sections in a cryostat at –20°C. The sections were mounted on egg albumin coated glass slides and dried with cold air. The dried sections were rinsed several times in sucrose buffer and twice in the buffer used for preparation of the incubation medium. For demonstration of acid phosphatase (AcPase) activity, the tissue sections were incubated 90 min at pH 5.0 in the medium described by Gomori using β-glycerophosphate as substrate[1]. For thiamine pyrophosphatase (TPPase) activity, incubation was for 2 hr at pH 7.2 in the medium of Novikoff and Goldfischer, using thiamine pyrophosphate as substrate[10]. For incubations longer than 30 min, the medium was replaced with fresh medium halfway through the incubation. All incubations were performed at 37°C, and all substrates were obtained from Sigma Chemical Co. Following incubation, the tissue sections were rinsed several times in buffer, postfixed in 1% OsO_4 for 1 hr, and embedded as described above.

Ultra-thin sections cut with a diamond knife were stained with uranyl acetate and lead citrate. They were observed using a JEOL 100CXII electron microscope.

Preparation method for SEM specimens: The A-O-D-O method was used[17,18]. Under anesthesia, the rats were perfused with the fixative of a mixture of 0.5% glutaraldehyde and 0.5% paraformaldehyde in cacodylate buffer solution at pH 7.3. After perfusion, the tracheas were removed in the same manner as mentioned above and placed in fresh room temperature fixative for a total of 1 hr. The tracheas were then rinsed in the same buffer and trimmed into strips, each containing two cartilage rings. They were fixed with 1% OsO_4 in 0.1M phosphate buffer solution for 1-2 hr. After rinsing, the specimens were successively immersed in 25 and 50% dimethyl sulfoxide (DMSO) in water for 30 and 60 min, respectively. They were then frozen using liquid nitrogen and cracked through a tracheal ligament into two pieces using a razor blade and hammer. The cracked pieces were immediately replaced in 50% DMSO solution to thaw. After rinsing with phosphate buffer, they were transferred to 0.1% OsO_4 in buffer solution and left standing at 20°C for about 4-5 days in order to remove cytoplasmic matrices. The specimens were fixed again in buffered 1% OsO_4 solution for 1 hr.They were then treated with 2% tannic acid aqueous solution for 3 hr and then rinsed with buffer solution overnight. The specimens were then fixed with 1% OsO_4 for 1 hr and were dehydrated with ethanol, and critical point dried. Dried specimens were coated very lightly (about 3 nm in thickness) with platinum in an ion coater (POLARON E5150; Polaron Equipment Ltd.). The prepared specimens were then observed using a field emission scanning electron microscope (Hitachi S-800).

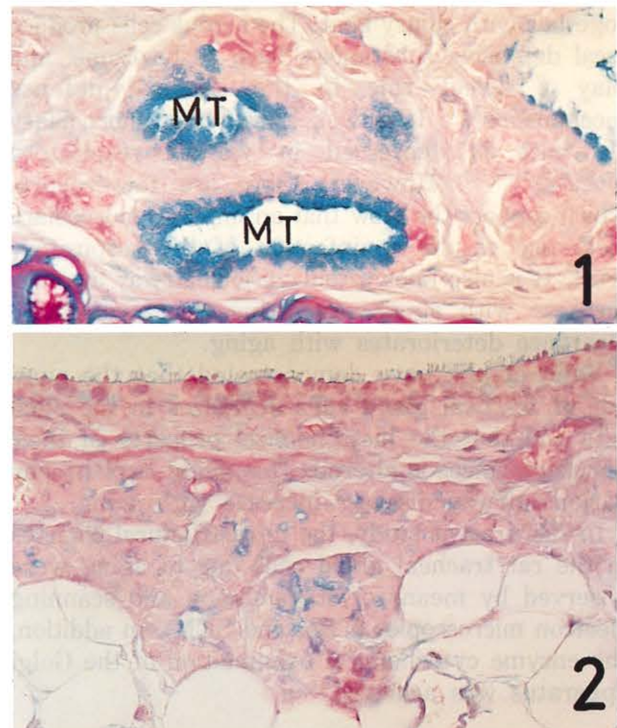
Light microscopy: At the time that tracheas were removed after perfusion with respective fixatives, a small part of the tracheal mucosa was isolated as a long strip over the total length and embedded in paraffin in the usual manner.Thin longitudinal sections were double stained with alcian blue, pH 2.5 (AB), and periodic acid Schiff (PAS), in order to demonstrate the presence of glycoprotein and mucopolysaccharide in the glandular cells, and to diagnose tracheal diseases.

RESULTS

Light microscopy

Rat tracheal glands were usually located in tracheal ligaments between neighbouring cartilage rings. The secretory units consisted of mucous tubules and serous acini in both young and aged rat subjects. In the young rats, mucous cells contained a large number of secretory granules which were stained blue by AB-PAS satining. Serous cells contained only PAS positive granules (Fig. 1). In the aged rats, mucous cells had granules which showed a similar staining pattern to that of the young animals. Yet, the serous cells contained both AB and PAS positive secretory grnaules in addition to the cells of only PAS positive granules (Fig. 2).

For histopathologically diagnosing a tracheal infection, all requirements were fulfilled for a 'clean



Figs. 1, 2. Light microscopic findings of rat tracheal gland stained with alcian blue, pH 2.5 (AB), and periodic acid Schiff (PAS). Tracheal gland consists of mucous tubules and serous acini in both young (Fig. 1) and aged (Fig. 2) rat subjects. In the aged rat, the serous cells contain both AB and PAS positive secretory granules, but those of young rat react to only PAS. MT: mucous tubule.

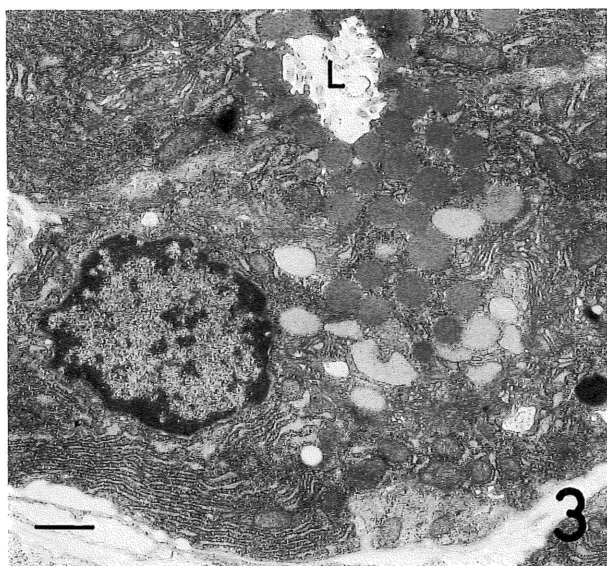


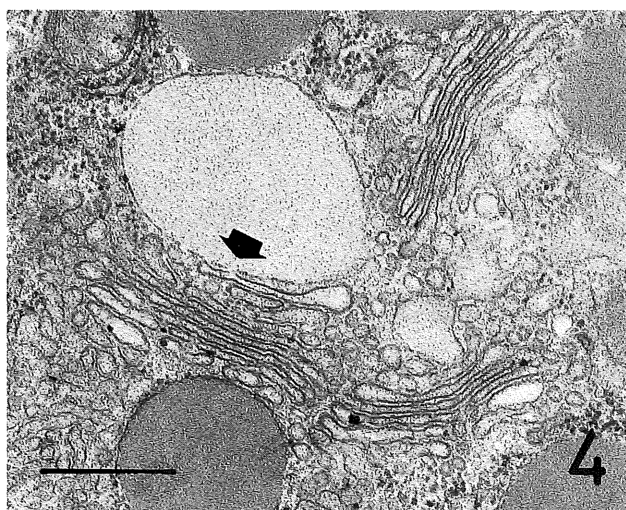
Fig. 3. Serous cells of the tracheal gland from a young rat. The cell contains many homogeneously dense secretory granules in the apical cytoplasm and abundant rough endoplasmic reticulum in perinuclear cytoplasm. L:acinar lumen. Bar:1μm.

trachea' showing no lymphocytic cuffing and a minimal goblet cell number.

Electron microscopy

Serous cells: In the young rats, tracheal serous cells contained many homogeneously dense granules in the apical cytoplasm, and abundant rough endoplasmic reticulum in parallel arrays in the perinuclear and basal cytoplasm (Fig. 3). The Golgi apparatus consisted of a stack of several saccules and many vesicles. Immature secretory granules of variable size and density were located near the *trans* face together with vesicles (Fig. 4). At the *trans* face of the Golgi stack, an innermost saccule which peeled off from the underlying Golgi cisterna branched out into membranous tubules approximately 100 nm in diameter (Fig. 5). These tubules again branched out but did not form a meshwork.The innermost saccule and the branched tubules constituted the *trans* Golgi network (TGN) and were referred to as a saccular element and a tubular portion, respectively.

In the aged rats, two types of serous cells were observed. Each type generally displayed a uniform granule population. The first type of serous cell contained homogeneously dense granules and had the same intracellular structure as seen in the young subjects. The second type of cell showed bizonal granules consisting of a moderately thick electron lucent cortex and a dense core (Fig. 6). The dense cores were spherical or irregular in shape. The electron density of the cortex was similar to that of the matrix of mucous cell granule (Figs. 6,13). In the Golgi region, immature granules which would become bizonal granules, exhibited various internal structures depending on their maturation phases (Fig. 7). Prosecretory granules connected with the TGN showed homogeneously electron lucent matrices. Immature granules which just left the TGN, showed irregular condensation of their contents. Many matured secretory granules contained an irregular core (Fig. 6). At the *trans* side of the Golgi apparatus of a cell with bizonal secretory granules, a large number of spherical and elliptical vesicles were seen (Fig. 7). The quantity of these vesicles was greater than that observed in the



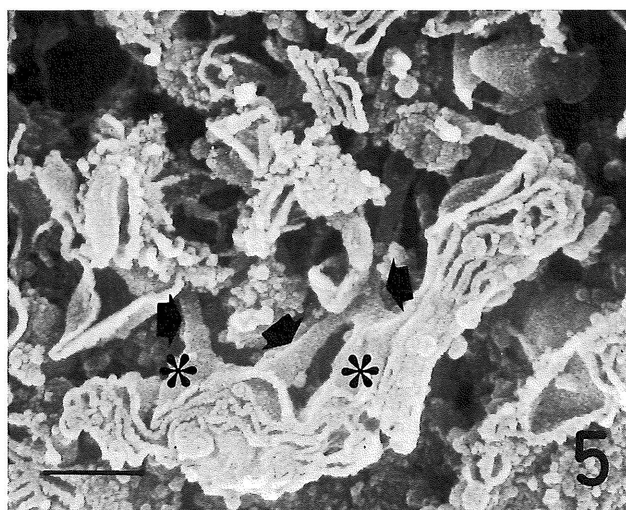


Fig. 4. Golgi region of a serous cell from the young rat tracheal gland. The Golgi apparatus consists of a Golgi stack and vesicles. At the *trans* face, an immature secretory granule is observed in connecting with the *trans* Golgi network (TGN) (arrow). Bar:0.5μm.

Fig. 5. SEM photograph of the Golgi apparatus of a serous cells in the young rat. The TGN consists of saccular elements (asterisks) and tubular portions (arrows). Each tubule of the tubular portion measures up to 100nm in diameter and branches out. Bar:0.5μm.

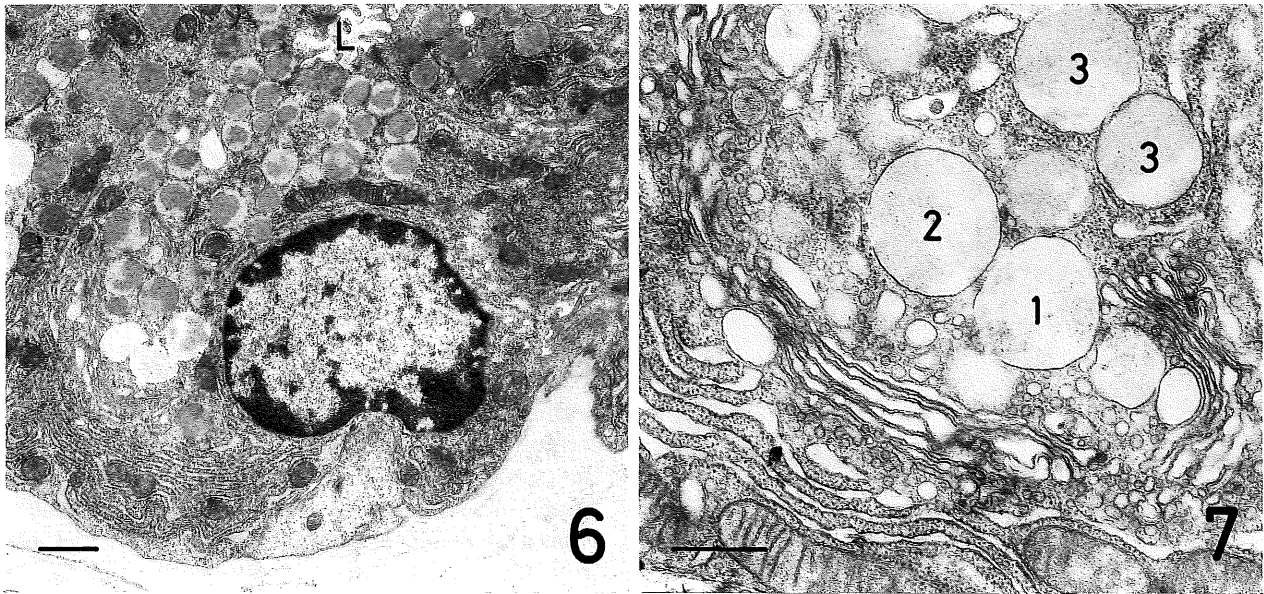
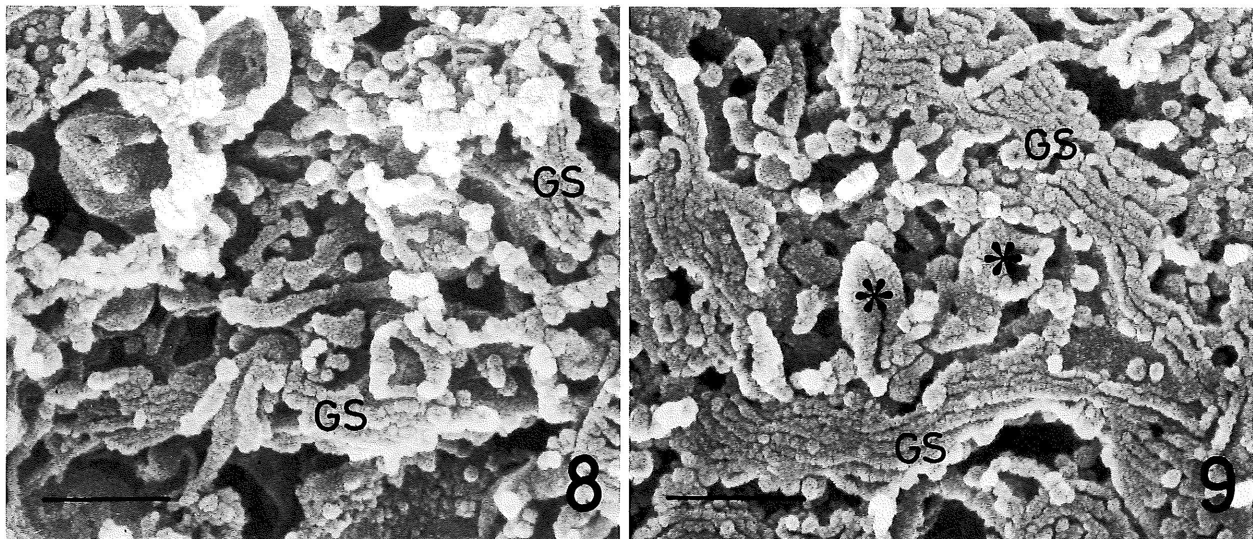


Fig. 6. Serous cells of the tracheal gland from an aged rat. Two types of cell are distinguished. The first type of cell contains homogeneously dense secretory granules (at the upper right and left of the figure), and the second type possesses bizonal granules (at the center of the figure). Rough endoplasmic reticulum is less developed than that of the serous cells of the young animal (see Fig. 3). L:acinar lumen. Bar:1μm.

Fig. 7. Higher magnification of the Golgi region of the aged rat serous cell which contains bizonal granules. In the *trans* region, a large number of vesicles are seen. Immature secretory granules exhibit various internal structures depending on their maturation phases (numbers, 1-3, indicate the order of their maturation). Bar:0.5μm.



Figs. 8, 9. Two higher magnification SEM photographs of the *trans* face view of the Golgi apparatus in the aged rat serous cells. The tubular portion of the TGN exhibits long branched tubules (Fig. 8) or a fine tubular meshwork with which prosecretory granules (asterisks) connect (Fig. 9). GS: Golgi stack. Bar:0.5μm.

young subjects. Rough endoplasmic reticulum in the serous cells of aged rats was somewhat less developed than that of young rats (Fig. 6). The tubular portion of the TGN exhibited long branched tubules (Fig. 8) or a fine tubular meshwork with which prosecretory granules were connected (Fig. 9). Upon enzyme cytochemical examination, AcPase was localized in both the saccular element and tubular portion of the TGN. The AcPase positive tubular portion showed a fine meshwork (Fig. 10) as seen in an SEM photograph (Fig. 9). On the other hand, TPPase reaction products were present only in a few *trans* Golgi saccules but not in the TGN (Fig. 11).

Mucous cells: In both young and aged rats, mucous cells contained many electron lucent secreto-

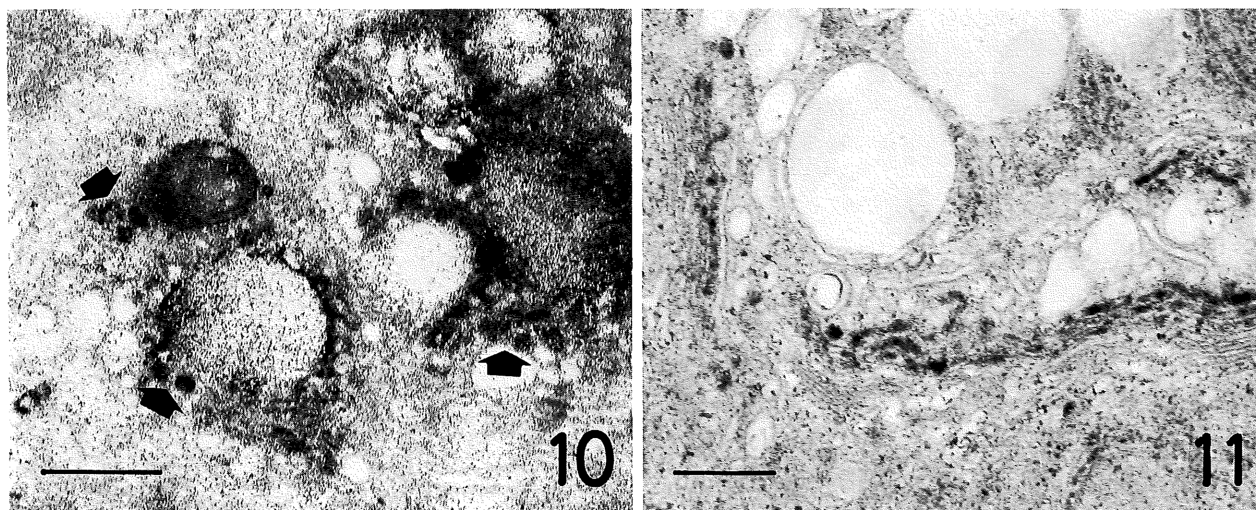


Fig. 10. The aged rat serous cell incubated for AcPase. Reaction product is present in the TGN and immature secretory granules. The AcPase positive tubular portion exhibits a fine meshwork (arrows). Bar:0.5μm.

Fig. 11. The aged rat serous cell incubated for TPPase. Reaction product is restricted to *trans* Golgi saccules. The TGN and bizonal immatured granules are not reactive. Bar:0.5μm.

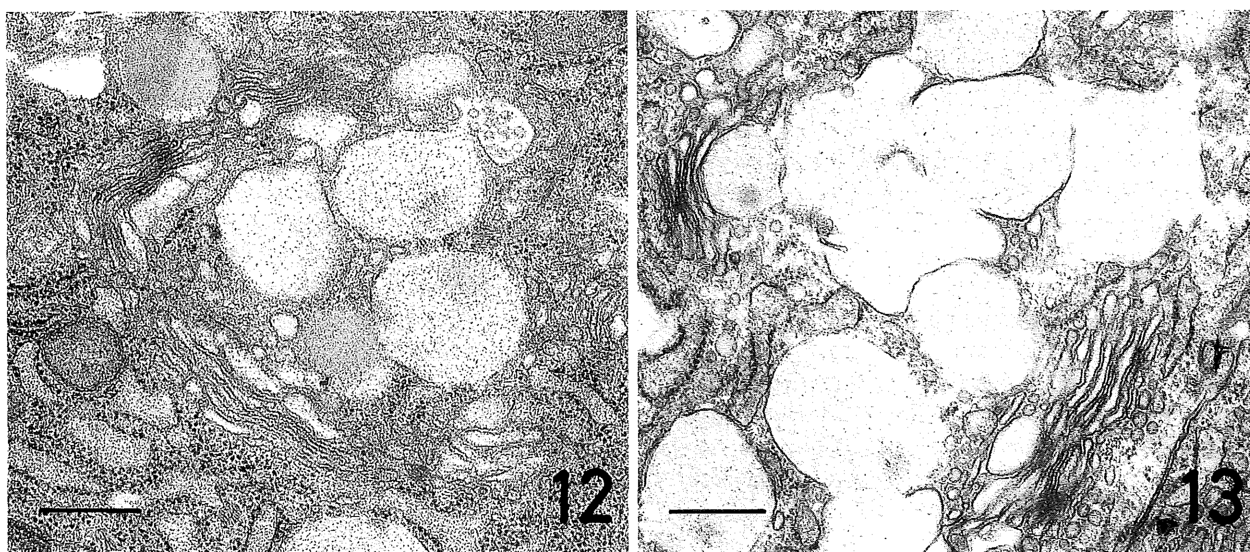


Fig. 12. The young rat mucous cell. Its electron lucent secretory granules contain a small dense core, and tend to fuse with each other. Bar:0.5μm.

Fig. 13. The aged rat mucous cell. Ultrastructural feature is the same as that of the young subject (see Fig. 12). GS: Golgi stack. Bar:0.5μm.

ry granules with a small dense core. These granules tended to fuse with each other (Figs. 12, 13). In SEM photographs, the TGN of both young and aged subjects showed branched tubules which formed an undeveloped meshwork (Figs. 14, 15). Other subcellular structures were also similar between both subjects.

DISCUSSION

The findings of this study revealed marked differences in the tracheal serous cells between young (8-17 week-old) and aged (20-30 month-old) rats. In the aged subjects, the serous cells contained bizonal granules which consisted of an irregularly shaped electron dense core and an electron lucent cortex. The electron density of the latter resembled that of mucous granule. The TGN was developed and formed a fine meshwork profile.

Mucociliary clearance in the trachea is one of the non-specific, host defense mechanisms which keep the airways clean. Mucociliary clearance is governed by three factors; cilia, mucus, and their interaction. A defect of anyone of these governing factors causes mucociliary dysfunction[16]. Mucus

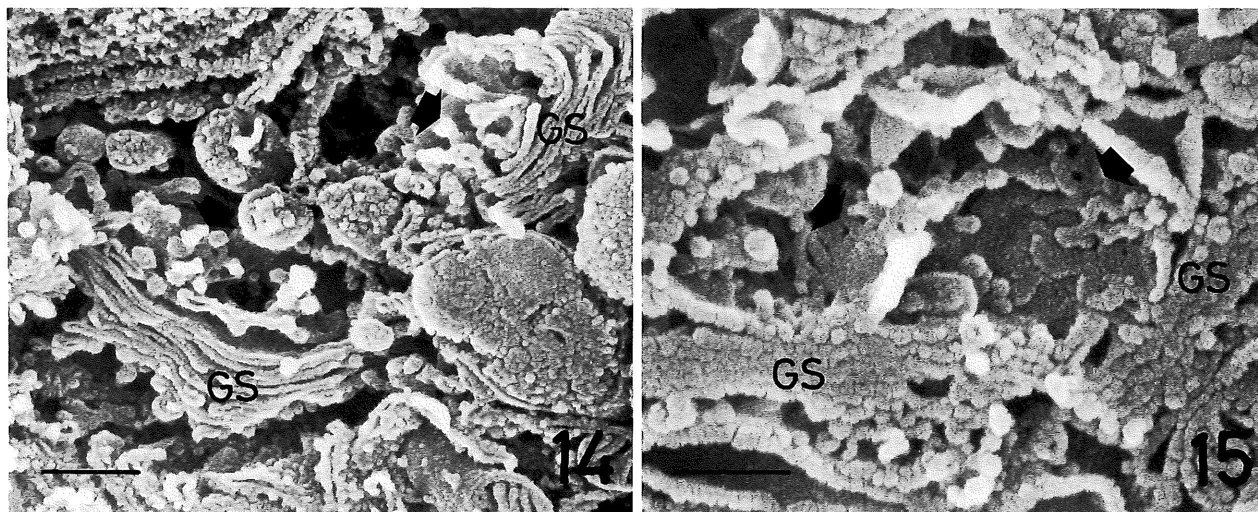


Fig. 14. SEM photograph of the Golgi apparatus in the young rat mucous cell. The tubular portion of the TGN forms a poorly developed meshwork (arrows). GS: Golgi stack. Bar:0.5μm.

Fig. 15. SEM photograph of the Golgi region in the aged rat mucous cell. The tubular portion of the TGN forms a meshwork (arrows) but is undeveloped in comparison with that of the aged rat serous cell (see Figs. 8, 9). GS: Golgi stack. Bar:0.5μm.

forms a mucous blanket which covers the respiratory tract and is conveyed upwards by ciliary beat. Rheologic study of the mucous blanket has developed a 2-layer concept, that is, the upper gel layer and the lower periciliary sol layer. The gel layer consists of high viscous mucus which is secreted from epithelial cells, including goblet cells, and from submucosal gland cells. The periciliary sol layer has a low viscosity and might contain pulmonary surfactant[7,22].

The main factor influencing mucociliary clearance is viscoelasticity of the gel layer which closely depends on biochemical components such as sialomucins and secretory proteins. The negatively charged sialyl and sulfuric residues can bind cations, such as calcium, and result in an increase in viscosity[9]. In the present study, ultrastructural alteration of the secretory granules was observed in the serous cells of the aged animal. This fact suggests a change in the property of the gel layer. Recently, Yajin et al[21] have demonstrated that sialic acid exists in the secretory granules of the aged rat serous cells, and that their secretory material is predominantly mucous glycoprotein. Their results indicate that the bizonal granule observed in the present study contains mucous glycoprotein with abundant sialic acid attached to its outer chain. A moderate amount of protein and plasma glycoprotein is also contained in this granule. The serous cell of the submucosal gland secretes proteins including immunoglobulins (secretory IgA), lysozyme and lactoferrin, and plays an important role in the host defense mechanism of the tracheobronchi[2]. Therefore, a decrease in the protein content of serous cell granules potentially leads to a reduction of the host defense mechanism.

In protein secreting cells, the Golgi apparatus is the most important organelle for formation of secretory granules. Kasuga and Harada[5,6] have recently confirmed the morphological and cytochemical dynamics of the Golgi apparatus of the tracheal serous cells. The TGN which belongs to the Golgi apparatus and consists of a tubular portion and a saccular element, is the real formation site of secretory granules, but the Golgi stack is not. The Golgi apparatus is involved in glycosylation and the addition of terminal sugars to the protein core[15]. Rothman[15] divided Golgi saccules into three compartments, *cis*, middle and *trans*, and clarified the localization of terminal glycosyltransferases including N-acetyl-D-glucosaminyl-, fucosyl-, galactosyl- and sialyltransferases. N-acetyl-D-glucosaminyl- and fucosyltransferases are localized in the middle compartment[8,9]. Galactosyltransferase exists in the *trans* compartment which is also positive to thiamine pyrophosphatase (TPPase)[13]. The *trans* saccule has been regarded as the localization site of sialyltransferase. However, according to a recent study, sialiltransferase is localized in the TGN where sialic acid is also present[14]. The present study showed that the TGN, which was only acid phosphatase positive, was well developed and formed a fine meshwork appearance in the aged rat serous cells. In the young animals, however, the TGN was branched and had only shorter tubules. This not only supports the recent findings of Yajin et al[21] on the appearance of sialic acid in serous secretory granules, but also suggests that the viscoelasticity of the gel layer increases with aging. Poor development of rough endoplasmic reticulum (Fig.6) in which the polypeptide core of the glycoprotein is synthesized under genetic control

may also support this assumption.

On the other hand, in the mucous cells where mucous glycoprotein is mainly formed, the degree of TGN development was not different between the young and the aged subjects. In both subjects, the TGN formed a meshwork indicating that secretory materials are rich in sialic acid. The TGN of the aged rat's serous cells showed more development than that of the mucous cells in which sialic acid predominates. The development of the TGN in serous cells might also be related to enhanced formation of secretory granules to compensate for a decrease in serous cell number[21]. In fact, the TGN of stimulated serous cells were more developed than that of un-stimulated ones at the time of reformation of secretory granules induced by pilocarpine[5,6].

TPPase was localized in a few *trans* Golgi cisternae of serous cells in both young and aged subjects. Roth and Berger[13] have demonstrated that galactosyltransferase is localized in this compartment and have suggested a concerted action of both galactosyltransferase and TPPase in chain elongation of maturing glycoprotein. This indicates that the site where galactose was added to glycoprotein might not be altered in extent. Further investigation is needed concerning the distribution of sugars in the Golgi apparatus.

The present study demonstrated marked alteration of the glycoprotein property of serous cells. It is well known that various airway diseases including chronic bronchitis and cystic fibrosis induce an increase in secretions of high viscosity. These phenomena probably result from hyperplasia of the goblet cells and the submucosal gland, and change of their mucous composition. There are some difficulties in evaluating the influence of aging on the respiratory system because of its high susceptibility to infection.It is known that goblet cells of the rat trachea develop differently according to environments rapidly increasing in number after irritation[3,12]. In the present study, all rats were raised in a semi-clean condition. This may be the reason why no histopathologic change was observed even in the aged rat, although they were raised for a long period of more than 16 months. This animal model certainly provides useful information about the secretion mechanism and the influence of drugs in the older respiratory mucosa.

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