Electron microscopic studies of various cells in the alveolar wall of mice with special reference to spheroid alveolar epithelial cells after intravenous injection of squid-ink (sepia-melanin) solution

Kiichi Suwa*

*Okayama University,
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Abstract

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ELECTRON MICROSCOPIC STUDIES OF VARIOUS CELLS IN THE ALVEOLAR WALL OF MICE WITH SPECIAL REFERENCE TO SPHEROID ALVEOLAR EPITHELIAL CELLS AFTER INTRAVENOUS INJECTION OF SQUID-INK (SEPIA-MELANIN) SOLUTION

Kiichi Suwa
Department of Anatomy, Okayama University Medical School, Okayama 700, Japan (Director: Prof. N. Otsuka)
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Abstract. The effect of an intravenous injection of squid-ink (sepia-melanin) solution on adult mouse spheroid alveolar epithelial cells was observed by the electron microscope. Sepia-melanin particles were seen in all alveolar wall cells examined that seems to suggest the entrance of sepia-melanin particles into the spheroid alveolar epithelial cells from the alveolar blood capillary. In cases of large penetrations of sepia-melanin particles into spheroid alveolar epithelial cells, a greater increase was found in the intramitochondrial granules. In addition, the so-called inclusion body believed to be formed by the degeneration of mitochondria had very high electron density and its quantity was abundant. On the contrary, in cases where the quantity of sepia-melanin entrance into the spheroid alveolar epithelial cell was small, neither an increase of intramitochondrial granules, an increase of the electron density nor an increase in the quantity of specific inclusion body was found.

It is known that the constituent cells of the mouse alveolar wall are alveolar epithelial cells, alveolar septal cells and endothelial cells of the alveolar blood capillary. The alveolar epithelial cells are of two kinds, as described by Karrer (1): one is the flat alveolar epithelial cell and the other is the spheroid alveolar epithelial cell. Various other terms have been used but the former cell has generally been called the alveolar epithelial cell of the small type and the latter cell as the alveolar epithelial cell of the large type.

According to Ham (2), the flat alveolar epithelial cells are called squamous epithelial surface cells, and the spheroid alveolar epithelial cells are called the secretory epithelial surface cells. In this paper the alveolar epithelial cells will be divided into two kinds, namely, flat alveolar epithelial cells and spheroid alveolar epithelial cells, according to morphology.

The alveolar septal cell is, needless to say, contained in the connective tissue of the alveolar septum, and it extends a slender projection along the alveolar wall. It is a mesenchymal cell that contains a variable number of lipid droplets in the cytoplasm.
Electron microscopically, the cytoplasm of the spheroid alveolar epithelial cell shows specific inclusion bodies of high electron density, but the nature of such inclusion bodies has been unclear (3). As early as 1923, Faure-Fremiet and Dragoiu (4) detected sulfur compounds in the alveolar epithelial cells of sheep, guinea pig and rat, and they designated such cells as granulocytes. In histochemical investigations of spheroid alveolar epithelial cells, the author (5) found some organic sulfur compounds of OSO₃H, S-S, and S-H radicals in the cytoplasm of these cells and detected particles that stain positively to Alcian blue, PAS and Irisolecht B-B-N. More recently the author (6) pointed out that such inclusion bodies are degenerate forms of mitochondria, because not only normal mitochondria but also specific inclusion bodies in the spheroid alveolar epithelial cells take up radioactive substances abundantly when agents, such as ³H-thymidine, ³⁵S-H₂SO₄ or ³⁵S-DL-cysteine are injected into the peritoneal cavity of mice, as observed by electron microscopy, as well as by electron radioautography.

In the present study squid-ink was injected intravenously into mice to observe the behavior of mitochondria in spheroid alveolar epithelial cells, especially how specific inclusion bodies are formed, as well as to observe the migration of squid-ink particles in the alveolar wall and the manner of squid-ink particle uptake by various cells.

MATERIALS AND METHODS

The ink sack of fresh squids (Sepia esculenta) was incised. Squid-ink was removed and placed in a freeze dryer for about one-hour, and after sufficient drying it was ground into fine powder with a pestle and mortar. The ground powder of squid-ink was then diluted with physiological salt solution to 0.25%.

This solution (0.2 ml/g body weight) was injected into the tail vein of adult mice of both sexes, every other day for five injections. The animals were sacrificed one hour after the last injection, and small samples of the lung were prepared for examination. Control lung samples from normal mice were also prepared.

These lung specimens were prefixed with 5% glutaraldehyde solution adjusted to pH 7.4 with 0.1M phosphate buffer for 1.5-2 hours, then fixed with osmic acid diluted to 1% with 0.1M phosphate buffer for 1.5-2 hours. After fixation the specimens were dehydrated through an ethanol series and embedded in Epon 812. Ultrathin sections were double-stained with uranyl acetate and lead citrate by the method described by Reynolds (7). Observations were conducted with a Hitachi HU-11 electron microscope.

RESULTS

The size of squid-ink (sepia-melanin) particles used in the experiment
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measured 0.1-0.6 μ in diameter and most were about 0.35 μ (Fig. 1). The mitochondria matrix of spheroid alveolar epithelial cells of mouse lung used as the control hardly contained any round intramitochondrial granules measuring about 30 mμ in diameter (Figs. 2, 3).

Relationship between spheroid alveolar epithelial cells and squid-ink pigments (Figs. 4-10). The outstanding findings were that in the matrix of the mitochondria of the spheroid alveolar epithelial cells, numerous fine particles of high electron density began to appear (Figs. 4-6, 8) and that relatively big particles, namely aggregates of fine particles, were present in both normal and degenerated mitochondria (Fig. 4).

In these spheroid alveolar epithelial cells containing numerous fine particles in the matrix of normal mitochondria, the electron density of the specific inclusion body was extremely high (Figs. 4-6, 8). Moreover, in such inclusion bodies there were relatively big particles (0.1-0.3 μ in size) of extremely high electron density in the center (Figs. 4, 6) or in direct contact with the limiting membrane (Fig. 5). Such particles were as large as the squid-ink particles phagocytized in the cytoplasm of spheroid alveolar epithelial cells (Figs. 5, 6).

The squid-ink particles were observed in the cytoplasm of spheroid alveolar epithelial cells (Figs. 4-7), and they were sometimes enveloped by a limiting membrane (Figs. 4, 6, 10), or they existed in close relationship with lysosome (Figs. 5, 7).

A characteristic feature was present in the relationship of sepia-melanin to lysosome. Most lysosomes proliferating in the cytoplasm were converted to lysophagosomes where sepia-melanin particles were phagocytized (Figs. 4, 6-8). Moreover, these ingested particles were seen undergoing a dissociation of digestion and lysis by the enzymatic actions of lysosomes (Figs. 4, 5, 7).

Thereafter, macromolecules or aggregates of particles of sepia-melanin were liberated into the cytoplasm (Figs. 4-8, 10), some of which resembled the size and electron density of the fine particles that entered mitochondria through the mitochondrial membrane (Figs. 4, 5, 10), and sepia-melanin particles that had been further decomposed to still finer particles were scattered in the cytoplasm. In connection with this phenomenon, there was often observed multivesicular bodies as residual bodies of lysosomes (Figs. 6-8, 10).

On examination of specific inclusion body formation (no. 1-7) in the cytoplasm when normal mitochondria (no. 1) began to degenerate, cristae of mitochondria grew indistinct (no. 2), and then the mitochondria changed suddenly to a diffuse substance (no. 3) of high electron density. This substance then began to show transparent interstices (no. 4), and thus enlarged by swelling (no. 5). At this instance, the diffuse substance changed to a lamellar body or myelin-figure and the interstices gradually grew bigger and the inclusion bodies
gradually disappeared (no. 6), and became a complete vacuole in some instances (no. 7).

Such inclusion bodies were sometimes excreted outside the cytoplasm. In cases of squid-ink ingestion, the inclusion bodies formed were of much higher electron density as compared with normal inclusion body, and their numbers were also greater (Figs. 4–8).

On the other hand, in some instances lysophagosomes may form inclusion bodies by fusing with degenerated mitochondria or with degenerated pieces of mitochondria (Figs. 4, 7), but lysophagosomes hardly appeared to migrate into specific inclusion bodies. As mitochondria were often broken into pieces (Fig. 6), small inclusion bodies were apt to be mistaken for arising from lysophagosomes after degeneration and formation of lamellar bodies (Figs. 4, 6, 7).

Differing from the above findings, in spheroid alveolar epithelial cells, where the amount of squid-ink pigment was small, the intramitochondrial granules were absent or very sparse, and the electron density of the specific inclusion body was also the same as that of the control (Figs. 9, 10).

Sepia-melanin was seen not only in the cytoplasm but also in the nucleus of spheroid alveolar epithelial cells (Fig. 5). Such macromolecules or micelles of sepia-melanin in the nucleus seemed to be of the same substance as the large or fine particles in mitochondria (Figs. 5, 10). Since the size of the granules in the mitochondria were about 30 mμ, the size and electron density were almost the same as those particles found in the nucleus.

It is worthy to take special note of the micelles or macromolecules of sepia-melanin that were liberated and scattered from lysophagosome and to consider that the sepia-melanin particles entered into mitochondria and nucleus in the form of molecules.

Relationship between cells that form the alveolar wall and squid-ink pigments (sepia-melanin). Aside from the spheroid alveolar epithelial cells, sepia-melanin was found in the alveolar capillary endothelial cells (Figs. 4, 7, 9), the alveolar septal cells (Figs. 5, 6, 8), the flat alveolar epithelial cells (Figs. 4, 7) and in the stroma of the alveolar septal connective tissue layer (Figs. 4, 7, 9).

These observations suggest two possible pathways for the squid-ink pigments to reach the spheroid alveolar epithelial cell from the alveolar capillary endothelial cell, namely, (a) the injected squid-ink particles may be taken up by the endothelial cells of the alveolar blood capillary and enter into spheroid alveolar epithelial cells via alveolar septal cells or via the stroma of alveolar septum, or (b) the squid-ink particles may enter into spheroid alveolar epithelial cells via the flat alveolar epithelial cells.
Sepia-melanin is ultimately excreted from the spheroid alveolar epithelial cells or from flat alveolar epithelial cells into the alveolar space. The size of the squid-ink particles observed in the cytoplasm of the spheroid alveolar epithelial cells varied considerably, and it seemed that the large particles of squid-ink pigment were disassimilated by lysosome and became molecules or fine particles of sepia-melanin, and they were scattered in the cytoplasm.

**DISCUSSION**

According to Tanikawa and Akiba (8), squid-ink pigment (sepia-melanin) is a pigment belonging to the melanin series. This melanin is thought to be a combination of protein and sepia-melanin which probably become molecules or fine granules of sepia-melanin when decomposed. The chemical formula of melanin is 5, 6-dihydroxyindole-2-carboxylic acid, according to Kato (9).

When squid-ink pigment particles of 0.15–0.6 μ in diameter were injected into mouse, the pigments were ingested by alveolar capillary endothelial cells, and after leaving these cells the particles entered either into the stroma where the connective tissue of alveolar septum is located, or they entered into alveolar septal cells, and after being transported from the alveolar septum, they entered into spheroid alveolar epithelial cells. Furthermore, it is thought that from capillary endothelial cells they entered into spheroid alveolar epithelial cells via the flat alveolar epithelial cells. The squid-ink pigments that entered into the spheroid alveolar epithelial cells were usually found in the cytoplasm in bare form, but pigments ingested by lysosomes that proliferated in the cell became lysophagosome, as previously reported by Weissmann (10).

At this instance, the squid-ink pigments of sepia-melanin combining with protein were probably disassociated by the enzymatic actions of polypeptidase, ribonuclease, acid phosphatase and other agents of the lysosome, and when the pigments became molecules or fine particles of sepia-melanin, they were freed and scattered in the cytoplasm.

On the other hand, squid-ink pigments before reaching the spheroid alveolar epithelial cells from alveolar capillary endothelial cells were disassociated by lysosomes of the various aforementioned cells, and the particles sometimes became molecules or were finely divided.

Thus, when molecules of sepia-melanin enter into mitochondria by permeating through the mitochondrial membrane, esterification of sepia-melanin may take place by transfer of sulfate from adenosine-3'-phosphate-5'-phosphosulfate (PAPS), that is, activated sulfate (11).

In vital staining experiments with basic dye by the author (12), when the mitochondrial cristae of the spheroid alveolar epithelial cell were injured, no inclusion bodies were formed, hence mitochondria cristae must be involved in
this mechanism. Namely, elementary particles that are considered adhering onto the inner membrane of mitochondria, as reported by Green, Tzagoloff and Oda (13) and Stoeckenius (14) may play an important role in supplying chemical energy for carrying on sulfate activation. However, the chief component of the inclusion body seems to exist in the matrix, so that the sulfate activation and transfer reported by Lipmann (11) might be carried out in the mitochondria matrix.

The sulfuric ester compound of sepia-melanin may thus be formed in the matrix of mitochondria, and the ion transition reported by Lynn and Brown (15) may take place.

By liberating $\text{H}^+$ from such $-\text{OSO}_3\text{H}$ group, the $\text{H}^+$ in the matrix increases, then divalent cations, such as cytoplasmic $\text{Mg}^{++}$ and $\text{Ca}^{++}$ enter into mitochondria, while $\text{H}^+$ moves out of the mitochondria. During this period at one OH radical of sepia-melanin, there already occurs sulfuric esterification while at another OH radical, phosphoric esterification is taking place. As the result of such reactions, Mg- or Ca-compound of phosphate (16, 17) are deposited within the mitochondria matrix in the form of intramitochondrial granules.

Next, it is presumed that when the sulfate of PAPS, a donor, is transferred to a hydroxy-group, such as sepia-melanin, an acceptor, in order to produce ester by catalysis of transfer enzyme, the mitochondria become degenerated. Because of the highly hydrophilic nature of $-\text{OSO}_3\text{H}$, the inclusion body becomes suspended in the vacuole and in addition if the hydrolytic enzymes of both lysosome and ribosome enter into the inclusion body, the lamellar body is disassimilated and disappears, and only the vacuoles remain.

Under certain circumstances the phagolysosome becomes degenerate and is converted into very small inclusion bodies, but such phenomena rarely occur.

As mentioned above, aside from the infiltration of sepia-melanin into mitochondria, the squid-ink pigments are decomposed by phagolysosomes and converted into sepia-melanin in the form of molecules or fine particles and enter into the nucleus. Such molecules or fine particles exist in the nucleus as relatively large particles by aggregating with one another. This, as already reported by the author (18), is the reason believed responsible for molecules or fine particles of sepia-melanin, passing through the nuclear membrane pores.

The infiltration of sepia-melanin into the nucleus can also be observed in alveolar septal cells, aside from spheroid alveolar epithelial cells. However, the infiltration of sepia-melanin into the mitochondria can be observed only in the spheroid alveolar epithelial cell, so that this cell seems to be a specific excretory cell among the various alveolar cells.
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REFERENCES

Legends to Figures (1-10)

AL, alveolar space; Bm, basement membrane; Ca, alveolar blood capillary; Co, collagen fibers in the alveolar septum; E, elastic fibers in the alveolar septum; F, flat alveolar epithelial cell; G, Golgi apparatus; Ib, inclusion body; Ld, lipid droplets of alveolar septal cell; Ly, lysosome; M, mitochondria of spheroid alveolar epithelial cell; mY, multivesicular body; N, nucleus of spheroid alveolar epithelial cell; PLy, phagolysosome; Se, alveolar septal cell; sm, particles of sepia-melanin; Tj, tight junction of the capillary endothelial cell.

Fig. 1. Particles of sepia-melanin (squid-ink pigment) used in this experiment measured 0.1-0.6 \( \mu \) in diameter, mostly about 0.35 \( \mu \). \( \times 5,810 \).

Figs. 2, 3. Lung tissues of control mice. No intramitochondrial granules were observed in the matrix of mitochondria. No. 1-4 in the spheroid alveolar epithelial cell of Fig. 3 show the degeneration processes of mitochondria to inclusion bodies. Fig. 2, \( \times 8,930 \); Fig. 3, \( \times 8,900 \).

Figs. 4-10. The lung tissues after intravenous injection of squid-ink solution into the mouse tail. No. 1-7 in the spheroid alveolar epithelial cell show the transition from mitochondria to inclusion bodies. Arrows point to squid-ink particles.

Fig. 4. There are numerous, specific granules of high electron density measuring about 30 \( \text{m} \) in the matrix of the mitochondria of spheroid alveolar epithelial cell, as well as relatively large granules formed by aggregation of fine particles measuring about 120 \( \text{m} \) in diameter in normal and degenerated mitochondria (arrows). Particles about 120-700 \( \text{m} \) believed to be squid-ink pigments are observed with or without limiting membrane in the cytoplasm. On the left margin of the figure, aggregates of rounded sepia-melanin are observed in the alveolar septal cell (arrow). No. 1-7 in spheroid alveolar epithelial cells show the transition of mitochondria to specific inclusion bodies. It is to be noted that the electron density of such inclusion bodies is extremely high. \( \times 5,340 \).

Fig. 5. Granules of sepia-melanin are observed in the cytoplasm and nucleus of the spheroid alveolar epithelial cell (arrow). In Golgi field sepia-melanin is observed dissociating from lysophagosome. Attention is called to specific granules in mitochondria and inclusion bodies of extremely high electron density. In addition, granules of sepia-melanin are seen in the projection of the alveolar septal cell (arrow). \( \times 8,950 \).

Fig. 6. Large and small granules of squid-ink (sepia-melanin) are seen in the spheroid alveolar epithelial cell with or without limiting membrane (arrow), or as phagolysosome being phagocytized in lysosome (arrow). In the center of the specific inclusion body of no. 4 in the spheroid alveolar epithelial cell, aggregates of high electron density are seen. Also broken pieces of mitochondria (no. 2) are seen in the spheroid alveolar epithelial cell. \( \times 8,950 \).

Fig. 7. Sepia melanin particles are seen in the cytoplasm of spheroid alveolar epithelial cell, in the cytoplasm and nucleus of alveolar septal cell, in the cytoplasm of alveolar capillary endothelial cell and the cytoplasm of flat alveolar epithelial cell (arrows). The mitochondria of the spheroid alveolar epithelial cell are almost all undergoing degeneration, but no specific granules are seen in the matrix. \( \times 5,350 \).

Fig. 8. In spheroid alveolar epithelial cell phagolysosome (PLy, arrow), fine particles of sepia-melanin (arrows) that are considered lysed and dissociated are observed in the cytoplasm. Multivesicular body, specific granules in the mitochondria and inclusion body of high electron density can be seen. In the alveolar septal cell, relatively large squid-ink pigment particles are seen (arrow). \( \times 8,920 \).

Fig. 9. Squid-ink particles are seen in the endothelial cell of the alveolar blood capil-
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However, particles are scarcely seen in the spheroid alveolar epithelial cell. In such instances, no intramitochondrial granules are seen in the spheroid alveolar epithelial cell, and the electron density of the specific inclusion body is the same as that of the control. ×8,930.

Fig. 10. Aggregates of fine particles closely resembling specific intramitochondrial granules are being enveloped by a double membrane (arrow) in spheroid alveolar epithelial cell. A most likely interpretation is that particles of decomposed sepia-melanin remain in the lysosome as the final product of phagolysosome. ×8,930.
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