ELECTRON-CYTOCHEMICAL STUDIES ON THE DIFFERENTIATION OF MOUSE LUNG ALVEOLAR EPITHELIAL CELLS WITH SPECIAL REFERENCE TO CHANGES IN MITOCHONDRIA

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Changes in mitochondrial structures and cytochrome oxidase activities were electron-cytochemically observed in lungs of butylated hydroxytoluene (BHT)-administered mice. The cytochrome oxidase activity was demonstrated by the diaminobenzidine method, and changes in the mitochondrial sizes during the differentiation were measured by morphometric analysis.

BHT selectively damaged Type I cells, and then Type II cells differentiated to Type I cells. When Type II cells differentiated to Type I cells, the mitochondria were greatly reduced in size and number. The mitochondria in intermediate cells became slender and divided into smaller mitochondria. It is possible that the amount of cytochrome oxidase per mitochondrion decreased. Thus, these cytochemical approaches evidenced functional as well as ultrastructural changes that occurred during the alveolar differentiation.

Lung alveolar epithelium is composed of three-kinds of epithelial cells; Type I, Type II and Type III cells. The shape of Type I cells is squamous or flat. The thin cytoplasmic sheets elongate up to 100 μ m covering approximately 96% of the alveolar surface (37). Type I cells face the interstitial capillary endothelial cells via basal lamina forming a blood-air barrier. There seem to be only a few poorly developed Type I cell organelles. On cross sections of Type I cells, small mitochondria, Golgi apparatus and vesicular endoplasmic reticulum are sparse in the perinuclear regions. A few small mitochondria are scattered in the thin sheets of the elongated cytoplasm. Characteristics of Type I cells are the 60 nm diameter pinocytotic vesicles at both the apical and basal plasma membranes.

Type II cells are cuboidal cells functioning in the synthesis and secretion of alveolar surfactant. These cells comprise 60% of the alveolar epithelial cells, although they occupy only 4% of alveolar surface area. Typical ultrastructures of Type II cells are 10 nm wide microvilli on the apical cell surface, lamellar bodies and peroxisomes in the cytoplasm. The lamellar bodies are a kind of secretory vesicle surrounded by a single limiting membrane. The matrices primarily contain phospholipid dipalmitoylphosphatidylcholine and exhibit an osmophilic-electron

dense lamellar structure. The lamellar substances are secreted out of the apical cell surface into the alveolar lumen and form a part of an alveolar lining layer. The result is that it functions as an alveolar surfactant to minimize water surface tension. Peroxisomes are quite simple organelles seen in Type II cells. Their shape is usually round with an average diameter of about $0.2\ \mu\text{m}$. Sometimes they are elongated to $1\ \mu\text{m}$. Presence of the peroxisomes in Type II cells was first reported by Petrik (30). He also showed the ultrastructural continuity between peroxisomes and endoplasmic reticulum. Hirai *et al.* (21) have also observed the same continuity.

The third group composing the alveolar epithelium is Type III cells. These cells have thick microvilli, 200 nm in width, and, therefore, are called alveolar brush cells (26). It is presumed that Type III cells function as chemoreceptors, since some afferent nerve ends come in contact with those cells at their base (10, 17, 38). Thus, it can be said that those three kinds of alveolar epithelial cells have eventually developed into quite different shapes and physiological functions. The investigations here focus on the cell differentiation of alveolar epithelial cells.

Hijiya (16) reported that Type III cells in rats proliferated after the administration of bleomycin, and that their numbers tended to increase with age. With this exception, we have little knowledge of the differentiation of Type III cells. On the other hand, many authors have investigated the differentiation of Type I and Type II cells. Hirai *et al.* (19, 20) and Witschi *et al.* (39) have found that butylated hydroxytoluene (BHT), an antioxidant, causes selective Type I cell damage in pulmonary alveoli in mice. Consequently, this damage caused the Type II cells to change into Type I cells resulting in Type I cell regeneration. Such cell differentiation was also reported by several other authors (2, 8, 9). Cell differentiation like this has also been observed in the developing fetuses of several animals (14, 22, 23, 26, 33).

During our continuous studies on lung differentiation, cytochemical studies have been made on peroxisomes (21, 27) and pinocytotic vesicles (18). We believe that cytochemical techniques may expand our knowledge and understanding of cell differentiation mechanisms. Thus, in the present study, further ultrastructural and cytochemical approaches concentrate on the mitochondria to investigate the changes that occur during the cell differentiation from Type II cells to Type I cells after BHT administration in mice.

MATERIALS AND METHODS

Animals

Male SW, and SPF MCH(ICR) mice (multi cross hybrid mice: a gift of Japan Crea Laboratories Co., Ltd., Tokyo) weighing approximately 20 g were fed *ad libitum* laboratory chow and water.

BHT Administration

One group of 35 mice was given 400 mg/kg of BHT in corn oil, as described earlier (21). Control mice received only corn oil. Five experimental and five control mice were sacrificed at each point in the experiment, 1–7 days after injection. The experiment was repeated three times.

Electron Microscopy

The lung fixation and resin block preparation procedures were done according to Hirai *et al.* (21).

Cytochemistry of Cytochrome Oxidase Activity

In order to determine the cytochemistry of the cytochrome oxidase activity, the lung tissues were fixed for 10 min with 2% buffered glutaraldehyde followed by several washings with a cold phosphate buffer solution. Lung slices 0.1 mm thick were made with a "Microslicer" (Dosaka EM Co., Kyoto). Cytochrome oxidase activity was demonstrated using the method of Seligman *et al.* (35). Tissue slices were incubated for 1 hr at 37°C in a medium that contained 1 mg/ml 3,3'-diaminobenzidine tetrahydrochloride (DAB: Dojindo Pharmaceutical Chemical Lab., Kumamoto), 0.1 mg/ml catalase (C-100: Sigma Chemical Co., Ltd., St. Louis, Mo. USA), and 0.1 M phosphate buffer at pH 7.2. One millimolar potassium cyanide and 10 mM sodium azide were used as inhibitors. When the inhibitors were used, all specific activities in the mitochondria were completely suppressed. All incubated tissues were postfixed for 1 hr in a cold 2% osmium tetroxide solution and embedded in a Quetol 653 resin introduced by Kushida (25). For observation, a JEM 100C electron microscope (Japan Electric Co., Ltd., Tokyo) or an H-500 electron microscope (Hitachi Seisakusho Co., Ltd., Tokyo) was used.

Morphometry of Mitochondria

In order to measure the changes in the mitochondrial sizes during the differentiation of Type II to Type I cells, the areas of the mitochondrial sections were measured by taking electron micrographs. Film negatives were examined under the computer-controlled image analyzer, Luzex 500 (Japan Regulator Co., Tokyo) and the MB-16001 personal computer (Hitachi Seisakusho Co., Ltd., Tokyo). A detached section from the central portion which caused hazy mitochondrial conformation and thereby inaccurately indicated size was omitted from the measurement.

We measured the longitude length and the equator width. In order to calculate the total area of mitochondrial inner membranes, mitochondria were postulated as ellipsoids in which the longitude is $2a$ and the equator is $2b$.

The ellipsoid area was calculated from the formula:

$$S = 2\pi a^2 \left(1 - e^2 + \frac{\sqrt{1-e^2}}{e} \sin^{-1} e \right), \quad \text{where } e = \frac{\sqrt{a^2 - b^2}}{a}$$

Crista membranes were postulated as equatorial planes which distribute at equal intervals. Therefore, crista area is calculated as follows:

$$S(x) = 2\pi b^2 \left(1 - \frac{x^2}{a^2} \right)$$

where coefficient 2 means that a crista has two surfaces of enzyme-active membranes. The total area of the membranes per mitochondrion is:

$$S_T = S + \sum_x S(x_n), \quad \text{where } n = \begin{cases} 2m \cdots 2 \sum_x S(x) & (x: x_1 \cdots x_m) \\ 2m+1 \cdots S(0) + 2 \sum_x S(x) & (x: x_1 \cdots x_m) \end{cases}$$

RESULTS

Normal Mice

Mitochondria in normal Type II cells were usually oval or elongated (Fig. 1). The width varied from 0.39 to 0.78 μm (mean 0.56 μm), and the length was from 0.95 to 2.61 μm (mean 1.76 μm). Mitochondria had an average of 17 cristae per mitochondrion. Cytochrome oxidase activity was demonstrated on the mitochondrial inner membrane including cristae (Fig. 2) as an increase in electron density. In normal Type I cells, the usual mitochondrial shape was oval and very small (Fig. 3). The average sizes were 0.25 μm wide and 0.39 μm long. Distribution of mitochondria in Type I cells mainly occurred at the perinuclear region,

FIG. 1. A typical Type II cell facing alveolar lumen (AL). Lamellar bodies (LB) and mitochondria (Mt) are prominent. $\times 8,000$

FIG. 2. Cytochrome oxidase activity (arrows) of a normal Type II cell. $\times 12,000$

FIG. 3. A typical normal Type I cell with small mitochondria (arrow). End, endothelial cell; E, erythrocyte. $\times 8,000$

FIG. 4. Cytochrome oxidase activity (arrows) of a normal Type I cell. $\times 6,000$

the number being from 0 to 3. Distribution at the extended cytoplasmic region was never constant. Such cytoplasm generally included one or two mitochondria in a cross section, but often lacked them. Fig. 4 shows the minor distribution in the cytochrome oxidase activity of a Type I cell.

BHT-Administered Mice

On day 1, after the BHT injection, as shown in the inset of Fig. 5, Type I cells were destroyed and became detached from the basal lamina. On day 2, intact Type II cells began to change to Type I cells, as previously described (19, 20). Type II cells flattened and extended their cytoplasm to cover the denuded basal lamina (Fig. 5). They rejected unused cytoplasm including mitochondria in the formation of myelin figures or cytoplasmic protrusions, as described in our earlier studies. However, almost all of the mitochondria showed no change. Around the 3rd to 5th day, the denuded basement membrane was fully covered by the extended thin sheets of Type II cells (Figs. 6, 7). The changes in their mitochondria were obvious. Mitochondria were stretched into slender shapes and seemed to be divided into smaller units, the mitochondria in the thin cytoplasm were particularly small in comparison to those in the original perinuclear region (Figs. 7, 8). Cytochrome oxidase activity was also demonstrated in those elongated or fragmented mitochondria (Fig. 8). Fig. 9 shows a typical intermediate cell between Type II and Type I cells. This cell apparently indicates both characteristics of Type II cells, i.e., peroxisomes, and of Type I cells, i.e., extended cytoplasm and pinocytotic vesicles. Mitochondria in reduced size and number were scattered in the cyto-

FIG. 5. A damaged alveolus, day 2 after BHT administration. Basal lamina (BL) is denuded. A Type II cell (II) began to extend the cytoplasm (arrows). No change in mitochondria is remarkable. $\times 6,900$ Inset shows a selective destruction of a Type I cell (I). Mac, macrophage. $\times 4,000$

plasms. On day 7, it appeared that the alveolar septum had been covered by new Type I cells, in which small mitochondria were observed (Fig. 10). Peroxisomes often remained in these cells.

Morphometry of Mitochondria and of Cytochrome Oxidase Activity

Change in the mitochondrial size and number and in cytochrome oxidase activity is shown in Table 1. During the course of cell differentiation, mitochondrial size and number reduced in Type II cells. The number of cristae also decreased. Cytochrome oxidase activities were measured by an image analyzer. There seemed to be no difference in intensity of the electron density exhibiting activity between Type II and Type I cells. However, the mitochondrial sizes of these two cells were

FIG. 6. A transitional Type II cell, day 4 after BHT administration. Arrows indicate the direction of cytoplasmic extension. Mitochondrial profiles appeared slender and divided. $\times 6,900$

quite different. Therefore, the enzyme amount in a mitochondrion must be in proportion to the surface area of the inner membranes. Surface area totals of the boundary of the mitochondrial inner membrane and the crista membranes were then calculated $8.8 \mu\text{m}^2$ for normal Type II cells and $0.4 \mu\text{m}^2$ for Type I cells. According to the differences in the total areas of enzyme-active mitochondrial membranes, cytochrome oxidase activity per mitochondrion in Type II cells was approximately 20 times that of Type I cells.

DISCUSSION

Differentiation of Type II Cells to Type I Cells in Developing Fetuses

Differentiation of Type II to Type I cells has been proven in developing fetuses

FIG. 7. Day 3 after BHT administration. A Type II cell extends the cytoplasm to cover the capillary endothelium. Arrows indicate the direction of cytoplasmic extension. Note the reduction of the mitochondrial size there compared with that in the original cell body. $\times 7,000$

FIG. 8. Cytochrome oxidase activity of a transitional Type II cell. Arrows indicate the direction of the cytoplasmic extension where mitochondria appeared reduced in size. $\times 11,000$

FIG. 9. A typical intermediate cell (IMC), day 5 after BHT administration. This cell simultaneously possesses peroxisomes (P) of Type II cell, and pinocytotic vesicles (PV) and reduced-sized mitochondria of Type I cell. $\times 11,500$

FIG. 10. Cytochrome oxidase activity of a new Type I cell, day 7 after BHT administration. The activity is demonstrated in some small mitochondria. A peroxisome remains in the cytoplasm. $\times 15,000$

and in experimental animals. This mechanism is now the generally accepted view. Adamson and Bowden (3) reported that Type II cells in rats originated from undifferentiated glandular cells which were labeled with ^3H -thymidine at days 18–19 of gestation. Almost all of the alveoli were lined with Type II cells possessing

TABLE 1. *Changes in Mitochondrial Sizes and Cytochrome Oxidase Activity During Differentiation of Type II Cells to Type I Cells*

Alveolar Cells	<i>n</i>	Length* ¹ μm (=2 <i>a</i>)	Width μm (=2 <i>b</i>)	No./ Cell Section	Crista No./ Mito.	Total Area* ² μm^2	Cyt. Ox. Act./Mito.
Normal							
Type II	40	1.76 ± 0.51	0.56 ± 0.11	12.5 ± 4.1	17.0 ± 4.8	8.84	100
Type I	40	0.39 ± 0.27	0.25 ± 0.09	2.8 ± 1.9	1.7 ± 0.8	0.43	4.8
BHT							
Days 2–4							
Transit. Type II* ³	28	1.47 ± 0.48	0.38 ± 0.11	16.6 ± 8.6	8.3 ± 3.8	3.11	35
Days 4–5							
Intermed.* ⁴	32	0.44 ± 0.25	0.24 ± 0.04	4.5 ± 1.5	2.0 ± 1.4	0.48	5.5
Day 7							
New Type I	18	0.38 ± 0.15	0.20 ± 0.07	6.5 ± 2.1	2.0 ± 0.8	0.36	4.1

*¹: Length represents the longitudinal size of mitochondria, and width is vertical to it. (mean $\pm$ SE)

*²: Total area includes mitochondrial boundary inner membrane and crista membrane.

*³: Transitional Type II cells

*⁴: Intermediate cells

lamellar bodies at day 21. Thereafter, Type I cells differentiated. Tritium-thymidine was never incorporated into Type I cells. Kauffman *et al.* (22) also reported that Type I cells did not have reproductive capacity. Type I cells certainly act as end cells in alveolar cell differentiation. Kikkawa *et al.* (23, 24) and Heine (14) suggested similar differentiation mechanisms in developing rabbit lungs, and Mercurio and Rhodin (26) in developing cat lungs.

Differentiation of Type II Cells to Type I Cells in Culture

Geppert *et al.* (11) cultured Type II cells from adult rats on a floating collagenous membrane in a culture medium. The cells formed a monolayer and produced basal lamina upon contact with the collagenous membrane. The ultrastructures were well preserved and Type II cell characteristics were maintained. However, according to Diglio and Kikkawa (6), when Type II cells were directly inoculated onto plastic culture plates, the cells maintained their original ultrastructures for only 3 to 5 days before the cell shapes flattened accompanying the disappearance of lamellar bodies and reduction in mitochondrial sizes. These transformed cells were very similar to Type I cells.

Differentiation of Type II Cells to Type I Cells in Experimental Animals

The possible differentiation of Type II to Type I cells was successfully proven in the regenerating lungs of experimental animals which received toxic chemicals. Adamson and Bowden (2) treated rats with 100% oxygen, and Evans *et al.* (8, 9) used nitrogen dioxide and ozone. They observed Type I cell injuries subsequent Type II cell proliferation. The Type II cells were labeled with ^3H -thymidine. When Type I cells had regenerated, the silver grains seen in Type II cells were transferred to new Type I cells. Our findings in BHT-administered mice directly demonstrated Type II cell differentiation into Type I cells (18-21). After the initial Type I cell damage produced by BHT, cuboidal Type II cells tended to move and the cell shapes appeared to be more irregular. Then, Type II cells flattened and extended their cytoplasm to cover the denuded basal lamina. The transitional Type II cells decreased in cell organelles. Lamellar bodies disappeared from the cells, microvilli diminished and electron opacity decreased in the cytoplasmic background. In contrast to these reducible changes, new formation of pinocytotic vesicles was observed. From the present study, changes were also noted in mitochondria.

Electron-cytochemical Approaches

In spite of these electron microscopic observations that offer a great deal of evidence to such dramatic ultrastructural differentiation, no information on physiological functions was obtained. For this purpose cytochemical approaches may be useful.

Peroxisomes and catalase activity: Catalase activity (peroxidatic activity of catalase), a marker enzyme for peroxisomes, was electron-cytochemically demonstrated first by Petrik (30), followed by Schneeberger (32, 33), Eguchi *et al.* (7), Hirai *et al.* (21), and Moriguchi *et al.* (27). Schneeberger (33) demonstrated catalase activity in developing mouse lungs, and found the presence of catalase-positive peroxisomes in undifferentiated alveolar cuboidal epithelial cells at canali-

cular period, days 16–17 of gestation. During perinatal days 19–20, a few peroxisomes were observed in some of Type I cells. Hirai *et al.* (21) cytochemically observed pulmonary catalase activity in BHT-administered mice. When new Type I cells were regenerated after acute BHT intoxication, we could really demonstrate catalase activity in peroxisome remnants in these cells. Therefore, there was no doubt in the possible differentiation of Type II cells to Type I cells in mice.

On the other hand, the possibility of Type II cell differentiation to Type I cells in human lungs had been suggested only by Okada and Genka (29) and Okada (28). They had observed regenerating flattened alveolar cells possessing dense bodies in lung diseases. These dense bodies resembled lamellar bodies of Type II cells. The technique to demonstrate catalase activity was adequately utilized in human lungs in our laboratories. Moriguchi *et al.* (27) obtained biopsy materials from a patient of pulmonary proteinosis, and cytochemically demonstrated catalase activity in lung peroxisomes in some regenerating alveolar areas. Proliferation of Type II cells was prominent there, and some of the cells extended their cytoplasm to cover the denuded basal lamina. There were a new generation of pinocytotic vesicles on the surfaces of the cytoplasmic extension, strongly suggesting a Type I cell profile. Type II cells lack the vesicles generally. Catalase-positive peroxisomes were found in these unusual cells simultaneously. Accordingly, it was concluded that Type I cells in human alveoli originate from Type II cells as they do in experimental animals.

Pinocytotic vesicles and alkaline phosphatase: As described above, pinocytotic vesicles are one of the characteristic cell organelles of Type I cells. New formation of these vesicles in the intermediate cells between Type II and Type I cells was observed by Hirai *et al.* (20, 21) and by Moriguchi *et al.* (27). In order to certify this morphogenesis, Hirai and Ogawa (18) tried to demonstrate alkaline phosphatase activity in mouse lungs after BHT administration. Three to five days after BHT treatment, Type II cells which still possessed lamellar bodies appeared flattened, and alkaline phosphatase activity was demonstrated in the pinocytotic vesicles formed on the basal cell surfaces.

Mitochondria and cytochrome oxidase activity: Mitochondria are distributed among all mammalian cells with the exception of erythrocytes, so it may sometimes be inconvenient to utilize mitochondria as a marker organelle to be followed up during cell differentiation. However, as shown in the present study, mitochondrial size was at least modified during the differentiation of alveolar Type II to Type I cells. The reduction in mitochondrial size seems to be a fundamental change in the adaptation of cells to a new situation, such as the extension of cell bodies resulting in thin sheets of cytoplasm which occupy only narrow spaces. Observing the ultrastructural profiles of cross sectioned Type I cells, mitochondria are usually very small in size and number, and are sparsely scattered. Tierney (36) described Type I cells to be rather anaerobic and to receive life energy from glycolysis. The total amounts of respiratory enzymes are small in Type I cells. These mitochondrial situations may be favorable for Type I cells in the avoidance of oxygen toxicity, regardless of the blood-air barrier formation where Type I cells are always exposed to a large amount of oxygen.

The Significance of Alveolar Cell Differentiation

The term "cell differentiation" generally refers to a phenomenon in which

blastic cells develop in each individual cell with specific cell structures and physiological functions. The blastic cells are, therefore, undifferentiated cells and are also called stem or precursor cells. We can see a good example in the differentiation of blood cells. In contrast to these, the differentiation of Type II to Type I cells seems to be somewhat unique because Type II cells possess particularly differentiated cell structures and functions. The structures include a cuboidal cell body with microvilli, lamellar bodies, peroxisomes and so on, and functions in the synthesis and secretion of alveolar surfactant. Rosenbaum *et al.* (31) emphasized that the defense mechanism against oxygen intoxication may belong to a peroxisome function in Type II cells. On the other hand, Type I cells are squamous and are completely different than Type II cells. Although Type II cells are devoid of endocytotic activity, Type I cells exhibit a capacity for pinocytosis or endocytosis. Bignon *et al.* (4) reported that blood plasma albumin is transported from blood capillaries to the alveolar lining layer through Type I cell pinocytotic vesicles. Type I cells may also participate in the transfer of horseradish peroxidase that is injected into the blood to alveolar lumen (34), and in the alveolar fluid leakage in viral pneumonia (5). Heppleston and Young (15) and Gonzalez-Crussi and Boston (13) revealed the role of Type I cells in the absorption of high molecules dropped into the respiratory air ways. We therefore understand that alveolar Type II and Type I cells are each greatly differentiated cell types. Finally, it may be concluded that in the lung alveoli, a highly differentiated cell can differentiate into another type of highly differentiated cell.

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