Increased Oxidative Metabolism in Cow Tracheal Epithelial Cells Cultured at Air–Liquid Interface

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Airway epithelial cells cultured at the air–liquid interface possess highly differentiated functions and structures compared with the cells cultured under immersion. We examined the oxidative metabolism and glycolysis in cow tracheal epithelial cells on Days 3, 6, 10, and 13, cultured under three different conditions: (*1*) immersion culture on porous filters with apical and basolateral feeding (IM), (*2*) air-exposed culture on porous filters with basolateral feeding, i.e., air–liquid interface culture (AI), and (*3*) conventional immersion culture in plastic dishes with apical feeding (DI). Lactate production was less in AI than in IM and DI on Day 3 through Day 13, whereas cellular adenosine triphosphate content and basal O_2 consumption were greater. Ouabain-sensitive and ouabain-insensitive O_2 consumption, and the uncoupled O_2 consumption were also greater in AI. Cytosolic lactate dehydrogenase activities on Day 10 were lower in AI, whereas α-ketoglutarate dehydrogenase activities were higher. The increased oxidative metabolism in AI was more pronounced at the late phase of culture (Days 10 and 13). In contrast, glycolysis remained elevated during the experiment in IM and DI. These data suggest that (*1*) AI begins to promote oxidative metabolism from growth phase by the provision of adequate oxygenation, and then further shifts to oxidative metabolism with differentiation; and (2) apical feeding may be responsible for the disturbance of the development of the oxidative metabolism. **Kondo, M., J. Tamaoki, A. Sakai, S. Kameyama, S. Kanoh, and K. Konno. 1997. Increased oxidative metabolism in cow tracheal epithelial cells cultured at air–liquid interface. Am. J. Respir. Cell Mol. Biol. 16:62–8.**

Cell culture of airway epithelia is a basic technique for the study of cell biology and physiology in the airway. The degree to which epithelial cells proliferate and differentiate is affected by various conditions including the isolation procedure, cell culture substrate, and medium. Although several advantages allowed the cultured cells to approach the level of the native tissue, morphologic characteristics and cellular functions often differ from the native tissue. Whittcut and associates (1) developed a unique biphasic culture system, "an air–liquid interface culture (AI)," in which the epithelial cells are fed only from the basolateral side and exposed to the air at the apical side. The cells cultured in this system exhibit more differentiated structures such as cilia and secretory granules (2–5), as compared with the cells cultured under the conventional immersion condition. Moreover, the cells grown in AI possess the mucin-like high molecular glycoconjugates (2) and active ion transport function (3–5) which are at a level similar to that of the native tissues.

We hypothesized that AI might obtain energy more effectively to support the differentiated structure and function than immersion culture. Several reports have demonstrated that glycolysis is stimulated in cultured cells under still, immersed conditions in dishes, while oxidative metabolism declines. Stevens (6) theoretically predicted that a fluid layer overlying an aerobically active hepatocyte monolayer can be no greater than 0.34 mm in depth in order to provide an adequate oxygen supply. This situation can hardly be achieved under immersed conditions, because the cells are covered with excessive media. In contrast, AI on a permeable support can uptake enough oxygen because the cells are exposed to the air at the apical surface. Recent evidence suggests that bronchial epithelial cells in AI show increased oxygen availability and enhanced Na^+ absorption (7). Here, we studied whether culture conditions could affect oxidative metabolism and glycolysis in cow tracheal epithelial cells. As a result, the oxidative metabolism was increased in AI, suggesting that the differentiated function and structure in AI may be associated with the improved energy metabolism in the epithelial cells.

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Abbreviations: air–liquid interface culture, AI; α-ketoglutarate dehydrogenase, α-KGDH; adenosine triphosphate, ATP; immersion culture in plastic dishes with apical feeding, DI; ethylenediamenetetraacetic acid, EDTA; fetal calf serum, FCS; immersion culture on porous filters with apical and basolateral feeding, IM; lactate dehydrogenase, LDH; phosphate-buffered saline, PBS.

Materials and Methods

Cow tracheas were obtained at a local slaughterhouse and brought to the laboratory in ice-cold phosphate-buffered saline (PBS). Cells were isolated by protease as described. Briefly, strips of epithelium were pulled off the submucosal tissues and incubated at 4°C overnight in PBS containing 0.05% protease (type XIV; Sigma Chemical Co., St. Louis, MO). The next day, mechanical agitation released sheets of cells which were further dispersed by repeated aspiration in a 20-ml pipette. Viability was ~90% as assessed by trypan blue exclusion. Cells were pelleted (200 × *g*, 10 min) and suspended in a 50:50 mixture of Dulbecco's modified Eagle medium and Ham's F12 medium (GIBCO, Grand Island, NY) containing 5% fetal calf serum (FCS). The cells were cultured under three different conditions: (*1*) immersion culture on porous filters with apical and basolateral feeding (IM); (*2*) air-exposed culture on porous filters with basolateral feeding (AI); (*3*) conventional immersion culture in dishes with apical feeding (DI) (Figure 1). For IM and AI experiments, the isolated cells were plated at 2.5×10^5 cells per cm^2 onto polycarbonate inserts of 24-mm diameter, 0.4-μm pore size, and 10-μm thickness (Costar Transwell, Cambridge, MA) which had been coated with human placental collagen (20 $\mu g/cm^2$, collagen Type IV; Sigma). The next day, for the AI experiments, the apical medium was removed, and the cells were fed only from the basolateral side with 2 ml medium. The apical surface was covered with a small volume (< 100 μl) of medium by capillary phenomenon until reaching the confluence (~Day 5). For the IM experiments, the cells were fed from both the apical and basolateral sides with 2 ml each of medium which corresponded to 4.4 mm in depth at the apical surface. For the DI experiments, the isolated cells were plated on 35-mm plastic dishes which had been coated with human placental collagen (20 $\mu g/cm^2$) and fed with 2 ml medium which corresponded to 2.1 mm in depth at the apical surface (Figure 1). The medium was serum-free, hormonally defined medium which contained insulin (10 μg/ml), transferrin (5 μg/ml), hydrocortisone (0.36 μg/ml), triiodothyronine (20 ng/ml), epidermal growth factor (25 ng/ml), and endothelial cell growth supplement (7.5 μg/ml), all obtained from Sigma (8). The cells were maintained in an incubator under 20% O_2 and 5% CO_2 atmosphere at 37°C, and culture media were changed once every 2 days. To estimate cell numbers, cells were detached by treatment with 0.05% trypsin and 0.02% ethylenediamenetetraacetic acid (EDTA) in Ca^{2+}-free PBS. Enzyme digestion was terminated by the addition of FCS. The cells were counted by a hemacytometer.

Electron microscopic techniques have been described in detail elsewhere (9). In brief, the cells on porous filters in IM or in AI or the cells in DI were fixed with glutaraldehyde/osmium tetroxide and embedded in epon. Thin sections (with a silver interference color) were stained with lead citrate and uranyl acetate for transmission electron microscopy.

To assess glycolytic and oxidative metabolism of the cells on Days 3, 6, 10, and 13, the following measurements were made: (*1*) lactate production; (*2*) cellular adenosine triphosphate (ATP) content; (*3*) activities of the glycolytic enzyme, lactate dehydrogenase (LDH), and one of the mitochondrial enzymes, α-ketoglutarate dehydrogenase (α-KGDH); (*4*) oxygen consumption.

Samples of growth media were taken for the measurement of lactate production. Lactate concentration was measured by the lactate oxidase method as described by Bergermeyer (10).

Determination of cellular ATP content was performed upon extracts of the cultured cell sheets using the firefly luciferin–luciferase kit (Lucifenol-LU kit; Kikkoman Co., Chiba, Japan). ATP concentration in the samples was assessed with a CAF-110 luminometer, (Japan Spectroscopic

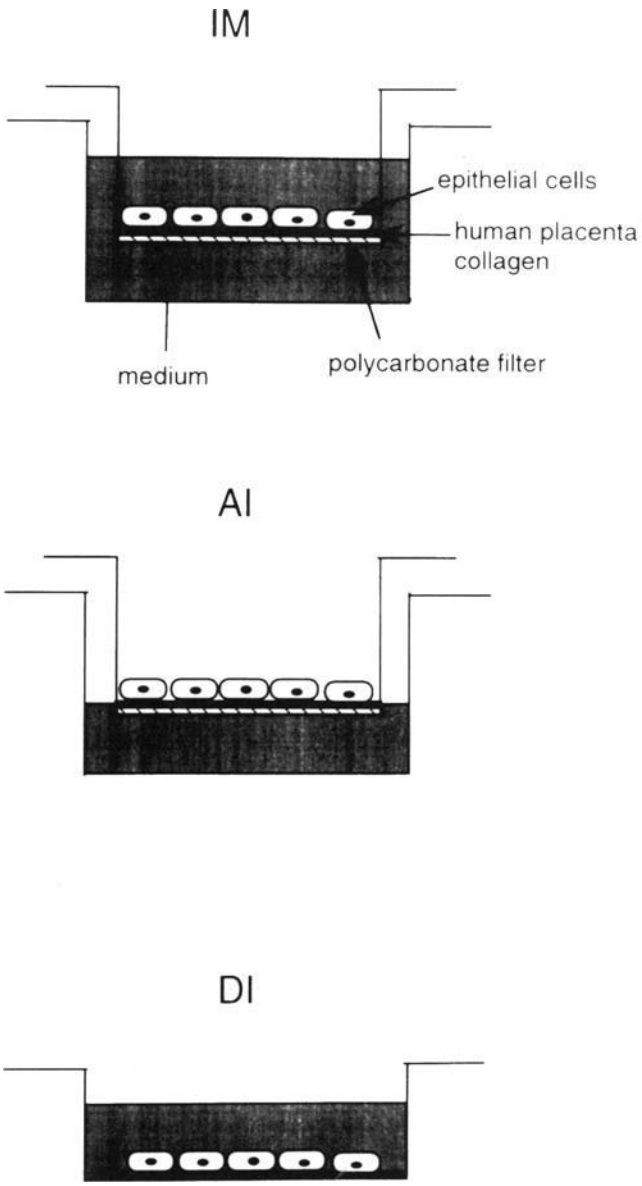

Figure 1. Schematic diagram of primary culture of cow tracheal epithelial cells. IM: immersion culture on porous filter with apical and basolateral feeding; AI: air-exposed culture on porous filter with basolateral feeding, i.e., air–liquid interface culture; DI: immersion culture in plastic dishes with apical feeding.

Co., Tokyo, Japan), where the standard curve was linear in the range of 10^{-10} to 10^{-7} M ATP.

For enzyme analysis, cultured cell sheets on filters were placed in 10 mM tris (hydroxymethyl) aminomethane (Tris) HCl (pH 7.4), containing 0.25 M sucrose, 1 mM EDTA, and 2 mM mercaptoethanol; then sonicated and centrifuged at 600 × *g* for 10 min after removing the filter. The supernatant was used to assay for α-KGDH and LDH activities. α-KGDH activities were determined by measuring the increase in adsorbance at 340 nm which was conversion of NAD to NADH in the presence of CoA and sodium α-ketoglutarate as described by Reed and Mukherjee (11). LDH activities were determined by measuring the change in absorbance at 340 nm resulting from the consumption of NADH in the presence of pyruvate. The protein was measured by the method of Lowry and colleagues, using bovine albumin as a standard (12).

Oxygen consumption by cultured cells was measured polarographically with a Clark-type oxygen electrode and a YSI oxymeter system (YSI 5300; Yellow Springs Instrument Co. Inc., Yellow Springs, OH). Cultured cell sheets were suspended in a sealed chamber maintained at 37°C and containing 4 ml Hank's balanced salt solution buffered with 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfornic acid (pH 7.4). Oxygen consumption was measured under the following conditions in series: (*1*) basal or unstimulated respiration; (*2*) the effect of ouabain (10^{-4} M; Sigma); (*3*) uncoupled respiration, the stimulated respiratory rate produced by 10^{-5} M of the mitochondrial uncoupler carbonylcyanide-*m*-chlorophenylhydrazone (Sigma).

Statistical Analysis

All values are expressed as means ± SE. Data were analyzed by analysis of variance, and multiple means were tested for significance using Fisher's protected lease significant difference test. Statements of significance were based on $P < 0.05$.

Results

Cell Growth

The growth of cells in AI was greater than that in IM or in DI (Figure 2). The cell number in AI was, for example, 3-fold higher than the others on Day 10 (AI: 16.0 ± 0.2 × 10^5 cells/cm^2; IM: 4.7 ± 0.7 × 10^5 cells/cm^2; DI: 5.0 ± 0.4 × 10^5 cells/cm^2; $n = 8$, $P < 0.01$ for AI versus IM, and AI versus DI). The protein content in AI was similarly 3-fold higher than that in IM or in DI on Day 10 (AI: 340 ± 7 μg/cm^2; IM: 110 ± 10 μg/cm^2; DI: 135 ± 14 μg/cm^2; $n = 8$, $P < 0.01$ for AI versus IM, and AI versus DI).

Morphology

The cells on Day 3 are shown in Figure 3. There was no significant morphologic difference between IM and AI conditions (Figures 3A and 3B). However, the cells in DI on Day 3 showed a more flattened appearance (Figure 3C). The cells in AI on Day 10 were multilayered and columnar-shaped. They showed highly differentiated characteristics, such as long microvili and many cilia at the apical membrane and abundant mitochondria in the cytoplasm (Figure 4B), whereas the cells in IM had short microvili, no cilia, and less mitochondria (Figure 4A). Similarly, the cells in DI showed a flattened and undifferentiated appearance (Figure 4C).

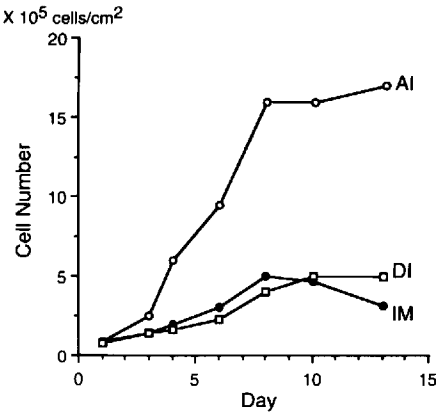

Figure 2. The growth curve of the cultured cells. Values are mean cell numbers of 3 to 4 experiments.

Lactate Production

Lactate production by the cells on Day 10 is demonstrated in Figure 5. The cells grown in IM showed a time-dependent production of lactate for 24 h. Most of the lactate was excreted to the basolateral side in IM (IM-apical side: 0.51 ± 0.11 mg/mg protein; IM-basolateral side: 3.26 ± 0.37 mg/mg protein; at 24 h, $n = 4$, $P < 0.001$). Lactate production in AI was significantly less than that in IM or in DI (AI: 0.66 ± 0.02 mg/mg protein; IM: 3.78 ± 0.38 mg/mg protein; DI: 2.14 ± 0.24 mg/mg protein, at 24 h, $n = 4$, $P < 0.01$ for AI versus IM, $P < 0.05$ for AI versus DI). Lactate production in DI seemed to be less than that in IM. Because the depth of medium in DI (~2.1 mm) was less than that in IM (~4.4 mm), we asked whether the smaller depth of the apical medium may cause the lower lactate production. As shown in Figure 6, lactate production in IM increased as the depth of apical medium increased. Therefore, lactate production of the cells in DI was not significantly different from that of the cells in IM cultured with ~2 mm depth of apical medium, suggesting that the depth of apical medium is an important factor for anaerobic metabolism whether or not the cells are cultured with basolateral feeding. As shown in Figure 7, the level of lactate production remained elevated during the experiment in IM and DI, whereas it was decreased with time in AI.

Cellular ATP Content

Cellular ATP content in AI was significantly greater than that in IM on Days 3–13, and that in DI on Days 6–13 (Figure 8). Cellular ATP content on Day 10 was 9.8 ± 0.3 nmol/mg protein in IM, 19.7 ± 2.5 nmol/mg protein in AI, and 14.1 ± 0.8 nmol/mg protein in DI ($n = 4$, $P < 0.05$ for IM versus AI and AI versus DI). Furthermore, cellular ATP content in AI increased with time.

Enzyme Activities

Cytosolic LDH activities of the cells in IM and DI on Day

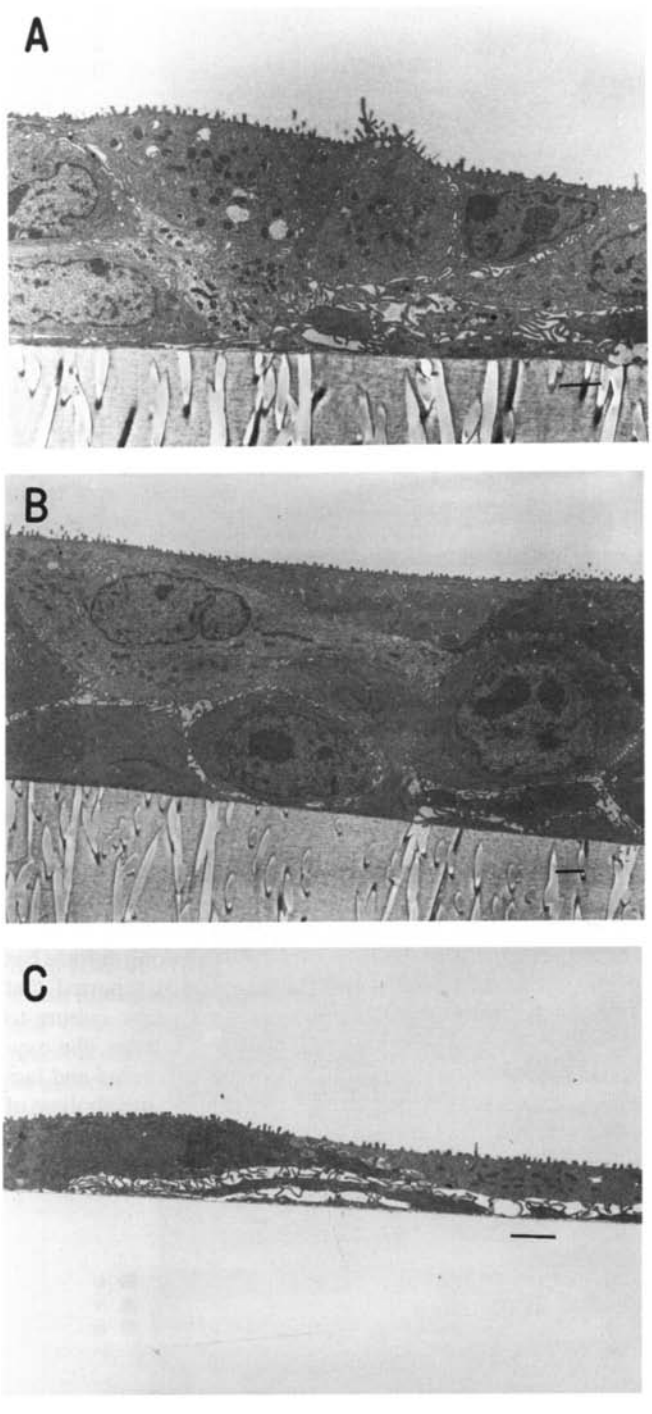

Figure 3. Transmission electron micrographs of cow tracheal epithelial cells cultured for 3 days. (*A*) Cells grown in IM; (*B*) cells grown in AI; (*C*) cells grown in DI. The morphology of the cells grown in AI was similar to that in IM, but the cells grown in DI had a more flattened appearance. Scale bar = 2 μm.

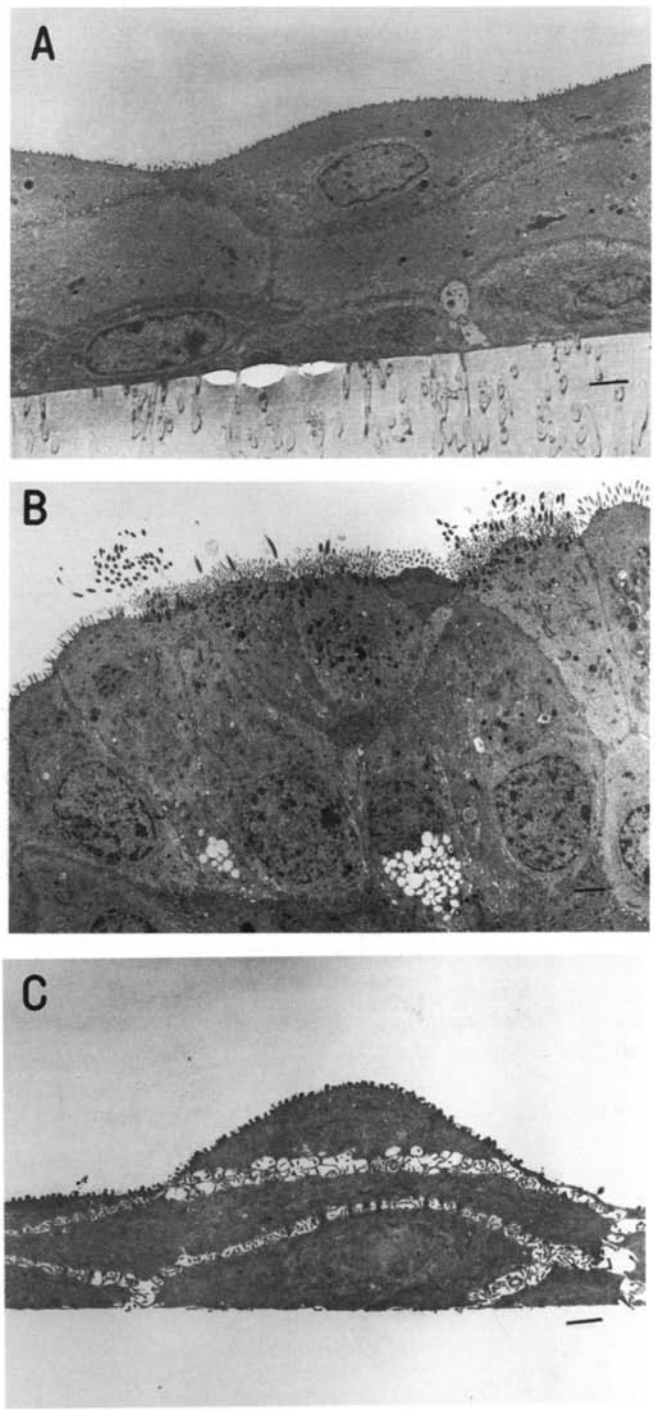

Figure 4. Transmission electron micrographs of cow tracheal epithelial cells cultured for 10 days. (*A*) Cells grown in IM; (*B*) cells grown in AI; (*C*) cells grown in DI. The cells grown in AI are columnar-shaped with the appearance of cilia, long microvili, and increased mitochondrial density. The cells grown in IM had no cilia and less mitochondria. Similarly, the cells grown in DI showed a flattened and undifferentiated appearance. Scale bar = 2 μm.

10 were significantly higher than those in AI on Day 10 (Table 1), whereas there was no significant difference on Day 3. LDH activities in IM and DI on Day 10 were significantly higher than those on Day 3. In contrast, α-KGDH activities of the cells in AI on Day 10 were slightly higher than those in IM on Day 10. Furthermore, α-KGDH activities of the cells in AI on Day 10 were higher than those in AI on Day 3.

Oxygen Consumption

The cells in AI showed elevated respiratory rate as compared with the cells in IM and DI. The baseline O_2 consumption in AI was higher on Days 3–13 than that in IM and DI (Figure 9). Furthermore, the baseline O_2 consumption in AI was

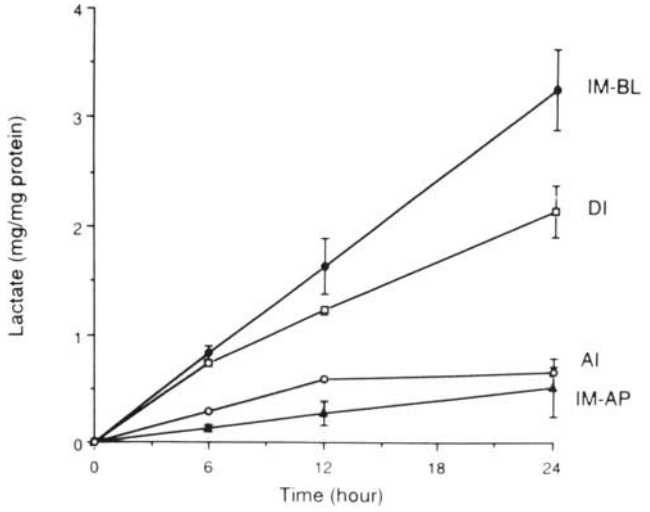

Figure 5. The time course of lactate production from the cells on Day 10 in IM, AI, and DI. *Closed circles*: lactate production to basolateral side in IM (IM-BL); *closed triangles*: lactate production to apical side in IM (IM-AP); *open circles*: lactate production in AI; *open squares*: lactate production in DI. $n = 4$ from different individuals.

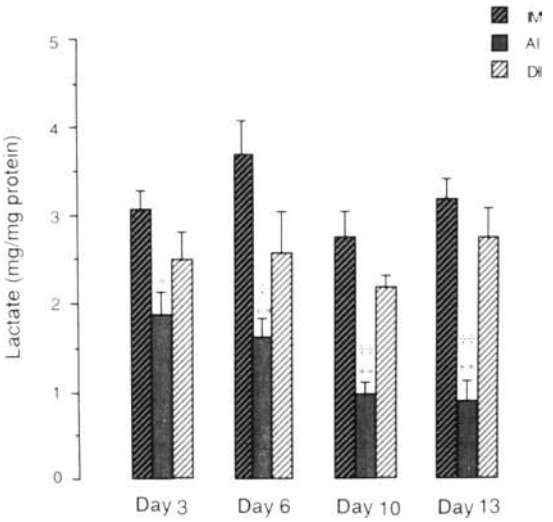

Figure 7. Lactate production from the cells grown in IM, AI, and DI on Days 3–13. Statistical analysis is performed between different culture conditions on the same day. $^{*}P < 0.05$. $^{**}P < 0.01$ for IM versus AI. $^{\dagger}P < 0.05$. $^{\dagger\dagger}P < 0.01$ for AI versus DI.

higher on Days 6–13 than on Day 3. The ouabain-sensitive and ouabain-insensitive O_2 consumption and the uncoupled O_2 consumption on Days 6–13 were higher in AI than in IM and DI (Table 2).

Discussion

In this study, the cells in AI exhibited increased oxidative metabolism as compared with the cells in IM and DI. This notion is based on the findings that AI showed decreased lactate production and less LDH activity as well as greater oxygen consumption and increased levels of intracellular ATP contents and α-KGDH activities. In contrast, the cells in IM and DI were more dependent on glycolysis. Stevens (6) reported that the cells cultured at static, immersed conditions might be deprived of oxygen, based on the direct measurement of oxygen tension at the surface of cultured cells and on the theoretical calculation of oxygen diffusion. In order to circumvent this problem, a rocking or shaking culture has been developed. Dickman and Mandel (13) reported that within 1 h after changing from immersed, static culture to shaking culture in primary renal proximal tubules, the oxygen consumption rates and ATP contents increased and lactate production decreased, suggesting that the metabolism of cells may immediately shift from glycolysis to oxidative metabolism. Moreover, the metabolism in immersed, static cul-

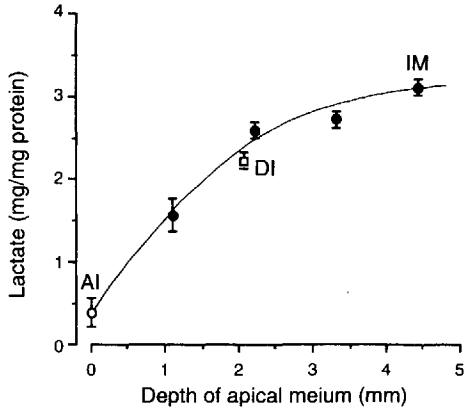

Figure 6. The effect of depth of apical medium on lactate production from the cells on Day 10. *Closed circles*: immersion culture fed with various depth of apical medium and 2-ml basolateral medium; *open circle*: air–liquid interface culture (i.e., ~0 mm in depth of apical medium) fed with 2-ml basolateral medium; *open square*: dish culture with 2-ml apical medium (~2.1 mm in depth). The lactate production was significantly correlated with the depth of apical medium ($P < 0.01$). $n = 4$ for each condition.

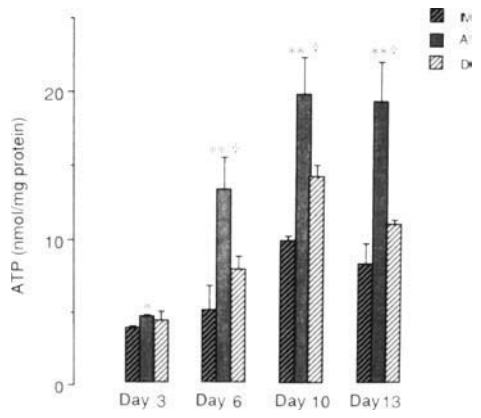

Figure 8. Cellular ATP content in the cells grown in IM, AI, and DI on Days 3–13. Statistical analysis is performed between different culture conditions on the same day. $n = 6$. $^{**}P < 0.01$ for IM versus AI. $^{\dagger}P < 0.05$ for AI versus DI.

TABLE 1
Enzyme activities in cow tracheal epithelial cells

		IM	AI	DI
LDH (1U/mg protein)	Day 3	3.8 ± 0.9	3.0 ± 0.6	2.9 ± 0.9
	Day 10	6.4 ± 0.5*	3.6 ± 0.5†§	5.1 ± 0.3*
α-KGDH (mIU/mg protein)	Day 3	3.0 ± 0.9	3.2 ± 0.5	3.4 ± 1.0
	Day 10	4.3 ± 0.4	5.5 ± 0.4*	4.8 ± 0.3*

Values are mean ± SE, $n = 5$.
* $P < 0.05$ for Day 3 versus Day 10.
† $P < 0.005$ for IM versus AI.
§ $P < 0.05$ for AI versus DI.

ture corresponded with the metabolism in shaking culture exposed to 1 to 3% O_2. Thus, immersed cells are living under hypoxic conditions with O_2 contents as low as 1 to 3%. Although AI resembles the rocking or shaking culture, AI seems to be more advantageous in terms of oxygen uptake because of continuous exposure to the air.

In this study, the increased oxidative metabolism in AI was apparent from the early growth phase of the culture (Day 3). This relatively rapid alteration in energy metabolism in AI is consistent with the report of the immediate shift to oxidative metabolism in the shaking culture (13). Murphy and associates (14) found that mitochondrial enzyme activities were higher in cultured cells grown under normoxic than under hypoxic conditions without alterations in DNA content, implying that O_2 may have a direct regulatory effect on mitochondrial enzymes.

Our data showed that the increased oxidative metabolism in AI was more pronounced at the late phase of this experiment (Days 10 and 13) than at the early phase (Day 3), because less lactate production, greater cellular ATP content, and more baseline O_2 consumption occurred at the late phase of culture. This alteration of energy metabolism may be associated with differentiation. In general, glycolytic enzyme activities are initially elevated during development and subsequently decline during differentiation (15). Moreover, α-KGDH activities in AI on Day 10 increased as compared with those on Day 3, suggesting that as oxygen availability changes, mitochondrial enzymes may be upregulated for adaptation. In contrast, LDH activities in IM and DI were higher on Day 10 than on Day 3, consistent with adaptation to prolonged hypoxia. Morphologic studies demonstrated that the cells in AI on Day 10 contained abundant mitochondria, suggesting that this elevated oxidative metabolism in AI may be related to the increment of mitochondrial mass. AI may select certain types of primary cells to grow, and the difference in oxidative metabolism at the late phase of culture may be due to this selection of different cell types. We cannot rule out this possibility, because the appearance of the cells in AI was extremely different from that in IM and DI. However, considering that the undifferentiated cells at the early phase of culture (Day 3) already demonstrated the improved oxidative metabolism, the change of metabolism probably precedes the differentiation. Furthermore, the rocking culture using the epithelial cell line LLC-PK1 also promoted oxidative metabolism as compared with static culture (16), suggesting that the growth of selected cell type may not be crucial for the change to the oxidative metabolism.

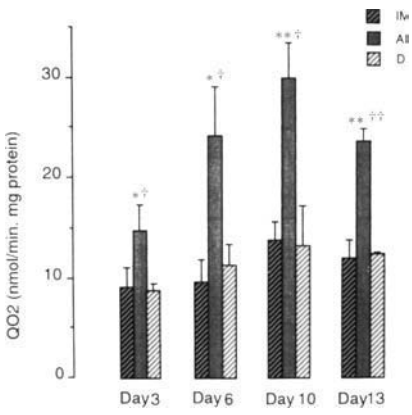

Figure 9. Baseline O_2 consumption (QO_2) in the cells grown in IM, AI, and DI on Days 3–13. Statistical analysis is performed between different culture conditions on the same day. $n = 8$. *$P < 0.05$. **$P < 0.01$ for IM versus AI. †$P < 0.05$. ††$P < 0.01$ for AI versus DI.

In this experiment, AI exhibited more intracellular ATP content than IM and DI. Sufficient energy supply may support cell growth and differentiation in AI. The cells in AI are known to have enhanced active ion transport, especially Na^+ absorption (7). The motivational force for active ion transport is the Na–K ATPase pump that is tightly coupled with mitochondrial respiration (17, 18). Because ouabain-sensitive O_2 consumption was higher in AI, increased ion transport in AI might be associated with stimulation of Na–K ATPase via increased oxidative metabolism. This is supported by the increase in mitochondria in electromicroscopic study and by the increase in uncoupled oxygen consumption for mitochondrial electron flow in AI.

Although the lactate production in DI seemed to be less than that in IM (Figure 5), metabolism of the cells in DI was not essentially different from that in IM as shown in Figure 6. Thus it can be speculated that apical feeding may disturb adequate oxygenation whether or not the cells are cultured with basolateral feeding.

Several reports have demonstrated that AI is an ideal model *in vitro* for studying airway epithelial function such as mucin secretion (2) and ion transport (3–5). Moreover, AI is likely to be a necessary requirement for the increase in

TABLE 2
O_2 consumption in culture of cow tracheal epithelial cells

	IM	AI	DI
Basal QO_2	9.6 ± 2.3	24.2 ± 4.9*†	11.6 ± 2.1
Ouabain-sensitive QO_2	4.0 ± 2.4	14.6 ± 3.9*†	4.1 ± 1.1
Ouabain-insensitive QO_2	5.6 ± 0.9	9.5 ± 1.5*	7.5 ± 1.0
CCCP-stimulated QO_2	25.6 ± 5.2	38.0 ± 2.4*	30.3 ± 6.6

Values are mean ± SE. n = 12. The cells were cultured on Days 6–13. QO_2 refers to nmol O_2/min.mg protein.
* $P < 0.05$, IM versus AI.
† $P < 0.05$, AI versus DI. 10^{-4} M ouabain or 10^{-5} M CCCP was added.

ciliogenesis in human epithelial cells *in vitro* (19). Although the mechanism by which AI improves differentiation has not been fully elucidated, our data suggest that differentiation in AI may be associated with improved oxygenation and increased energy metabolism.

In conclusion, the metabolism in AI was shifted from glycolysis to oxidative metabolism, whereas the metabolism in IM and DI was mainly dependent on glycolysis. Increased oxygen availability and ATP production may support highly differentiated function and structure in AI.

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