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Effects of vitamin A on proliferation of human distal airway epithelial cells in culture

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Abstract Using a serum-free culture method, we investigated the effects of vitamin A on the proliferation of human distal airway epithelial cells. Outgrowth of epithelial cells from lung tissue explants was enhanced by treatment with all-trans retinol at concentrations of 10^{-8} to 10^{-7} M. The colony-forming activity of cells harvested from the primary culture and replated onto Swiss 3T3 fibroblastic feeders was, in contrast, significantly reduced by 10^{-7} M to 10^{-5} M retinol. When the primary cells were harvested and subcultured on Primaria plates, population expansion was also inhibited by retinol at 10^{-10} to 10^{-6} M. We further investigated the cells to determine whether there was any difference in sensitivity to the growth-inhibitory effects of vitamin A between cells from the primary culture incubated with and without retinol. The population increase in cells harvested from the primary culture was inhibited equally in retinol-treated and non-treated cells by subsequent treatment with retinol or retinoic acid, this inhibition being dose-dependent. DNA synthetic activity was also inhibited. Interestingly, both the growth rate and the colony-forming efficiency on feeders were greater in the subculture of cells from the retinol-treated primary culture than in those non-treated. When the cells in the secondary subculture were treated with retinoic acid and replated again, they showed a greater population increase rate than those non-treated. Our results showed that human distal airway epithelial cells isolated from lung tissue were sensitive to the growth-inhibitory effect of vitamin A, but the proliferative potential in some fraction of the epithelial cell population was possibly enhanced by vitamin A treatment.

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Introduction

The distal airway is one of the most susceptible sites for injury to the lung and it is generally believed that peripheral lung cancers in human originate from alveolar and/or bronchiolar cells [26]. Knowledge of the regulatory mechanisms responsible for the growth and differentiation of epithelial cells in the human distal airway is thus of importance in addressing various aspects of pulmonary pathobiology and carcinogenesis. Most information on the growth and differentiation of respiratory epithelial cells in vitro, in both Man and animals, is derived from the culture of cells from proximal portions of the airway, such as nasal [30] and tracheo-bronchial epithelial cells [10, 11, 21, 28]. We have developed a culture system for studying the growth and differentiation of human distal airway epithelial cells [8]. In this system, the epithelial cells outgrown from the explants of human peripheral lung exhibit extensive proliferative capacity and have the potential to differentiate into the mucociliary or epidermoid phenotype. Small amounts of serum compel these epithelial cells to become ciliated cells and non-ciliated secretory cells [8].

Vitamin A is one of the diverse factors that contribute to the growth and differentiation of respiratory epithelial cells, and is important as a biological substance helping to maintain physiological conditions in respiratory epithelium [6, 14]. Lung cells express retinoic acid nuclear receptor signals [22], and various actions of vitamin A are exerted through these receptors [18]. Vitamin A deficiency produces squamous metaplasia in the respiratory epithelium both in vivo [1, 2, 14, 25] and in vitro [13]. While the retinoids, which are structural and functional analogs of vitamin A, have been shown to inhibit the proliferation of fibroblasts and of a variety of tumour cell lines in culture [12], the effects of vitamin A itself on the growth of airway epithelial cells seem to be com-

plex. Retinoic acid increases tritriated-thymidine labelling in epithelial cells derived from carcinogen-exposed rat trachea [9] and in hamster tracheal cells in collagen gel culture [16]. Retinoic acid promotes the expression of some protooncogenes related to cell growth [20]. In contrast, some investigators have reported that retinol suppressed the growth of epithelial cells in hamster trachea organ culture [23] and in collagen gel culture of a cell line derived from human fetal lung [5]. Chopra [2] demonstrated that retinoic acid inhibits the replication of basal cells and stimulates that of mucus cells in a trancheal organ culture derived from vitamin A-deficient hamsters. It has also been shown that retinoic acid suppresses the growth of human lung carcinoma cells in vitro [4]. There is no information on the effects of vitamin A on the growth and differentiation of human distal airway epithelial cells, and so, in the present study, using a culture system, we investigated the effects of vitamin A on the proliferation of human distal airway epithelial cells by evaluating population expansion rate, colony-forming activity, and DNA-synthesizing activity. We also examined the effect of vitamin A treatment of progenitor cells on the growth capacity of progeny cells.

Materials and methods

Bovine insulin, human transferrin, hydrocortisone, triiodo-thyronine (T3), all-trans retinol, all-trans retinoic acid, and dimethylsulphoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, Mo., USA). Epidermal growth factor (EGF), endothelial cell growth supplement (ECGS), and Nu-serum IV were obtained from Collaborative Research Inc. (Bedford, Mass., USA). Ham's F12 medium powder, Dulbecco's modified minimum essential medium powder, penicillin/streptomycin solution and trypsin-EDTA solution were purchased from Gibco (Life Technologies Inc., Gaithersberg, M.D., USA), and N-2-hydroxy-ethylpiperazine-N'-2-ethane-sulphonic acid (HEPES) and amphotericin B were obtained from Flow Laboratories Inc. (Maclean, Va., USA). Cholera toxin was obtained from the Chemo-Sero-Therapeutic Research Institute (Kumamoto, Japan), and fetal bovine serum (FBS) was obtained from Hyclone Laboratories Inc. (Logan, Ut). Rabbit anti-keratin polyclonal antibody was obtained from Seikagaku Co. (Tokyo, Japan) and mouse anti-bromodeoxyuridine (BrdUrd) monoclonal antibody from Becton-Dickinson (Mountain-View, Calif., USA). Plastic culture dishes (60 mm) and those with 2 mm grids were obtained from Corning (Iwaki Glass, Chiba, Japan). Twenty-four multi-well Primaria culture plates and 75-cm^2 flasks were from Falcon (Becton-Dickinson), 6-multiwell plastic culture plates were from Costar Co. (Cambridge, Mass., USA), and Lab-tek chamber glass slides (8 wells) were obtained from Nunc Inc. (Naperville, Ill., USA).

Ham's F12 medium was buffered with 15mM HEPES (pH 7.3) and supplemented with insulin (5 μg/ml), transferrin (5 μg/ml), T3 (2×10^{-10} M), hydrocortisone (10^{-7} M), EGF (10 ng/ml), ECGS (15 μg/ml) and cholera toxin (10 ng/ml). This defined medium also contained penicillin (50 IU/ml), streptomycin (50 μg/ml) and amphotericin B (2.5 μg/ml); it was used for culture of explants and primary epithelial cells derived from the distal airway of the human lung. Retinol and retinoic acid were dissolved in DMSO and stored at −20° C until use. The final concentration of DMSO in the culture medium was 0.1%.

The method used to obtain human lung tissue and to culture human lung explants were essentially the same as those described previously [8]. Briefly, normal lung tissue was obtained from the peripheral area of pulmonary lobes surgically resected for lung cancer. Immediately after the lung was removed, the subpleural lung tissue from areas with a gross normal appearance was sectioned with scissors. The fragments were stored in ice-chilled Ham's F12 medium containing antibiotics until required for the preparation of explants for culture. After the pleural tissues were removed, the fragments were then further sectioned into small pieces, approximately 0.5 cm cubic, with scissors. Three explants per dish were placed at sites scratched with a steel blade in a 60 mm culture dish. The cultures were incubated with the defined F12 medium at 37° C in a humidified atmosphere containing 5% carbon dioxide. The medium was changed every 2 or 3 days. To obtain primary epithelial cells outgrown from the explants, retinol (10^{-7} M) was added to the medium. For some experiments, the explants were incubated in medium containing 0.1% DMSO instead of retinol. The degree of epithelial cell outgrowth from lung explants was estimated in the 60 mm dishes with 2 mm grids where the 3 explants were placed; the number of grids occupied by the outgrown epithelial cells was counted at 3 weeks of culture under a phase microscope. Primary epithelial cells were harvested 2 to 3 weeks after the initiation of cultures by trypsinization. The cells were pooled and used for the following experiments.

In the colony-forming assay Swiss 3T3 cells were cultured in a 75 cm^2 culture flask with Dulbecco's modified minimal essential medium containing 5% FBS; at a semi-confluent state they were harvested by trypisinization. The cells were irradiated at 50 Gy from gamma sources (^{137}Cs), then plated in 6 multiwell plates at 3×10^5 cells per well. After overnight incubation in the defined F12 medium containing 0.5% FBS, the medium was aspirated, and the epithelial cells harvested from the primary explant cultures (see above) were seeded on the feeder layers, at 2×10^3 or 4×10^3 cells per well, in the defined F12 medium containing both 10^{-7} or 10^{-5} M retinol and various concentrations of FBS (0% to 5%). Seven days later, the cells were fixed in methanol after being rinsed with PBS and stained with a Giemsa solution. To confirm the epithelial nature of the cells, we stained some cultures by the indirect immunoperoxidase method, using a rabbit anti-keratin polyclonal antibody. Epithelial colonies consisting of 30 or more cells were counted and colony-forming efficiency (CFE) was calculated.

The growth of human distal airway epithelial cells was studied in terms of increases in the cell population on plastic substratum. We used the Primaria plates in this experiment because these provide excellent cell attachment to wells. Epithelial cells were replated at 1×10^4 to 4×10^4 cells per well in the defined F12 medium. Twelve to 24 h after seeding, when the cells were attached to the bottom of wells, the medium was aspirated, the cultures were washed with PBS, and fresh medium containing various concentrations of retinol was placed in the wells. At 5 and 7 days, the cells were dissociated by trypsinization and the cell number was determined in a haemocytometer. Another experiment was carried out to clarify whether vitamin A was involved in sustaining the growth capacity of human distal airway epithelial cells. For this purpose, primary epithelial cells were obtained from the explant culture with retinoic acid, and these were plated and cultured in the medium with or without retinoic acid for 7 days. The cells were then harvested, pooled, and further cultivated with 2% Nu-serum (equivalent to 0.5% FBS concentration) for 7 days. The cell number was determined at the end of the culture.

For assessment of the bromodeoxyuridine (BrdUrd) labelling index, the epithelial cells were replated onto Lab-Tek chamber glass slides at 1.5×10^4 cells per well. Twelve hours after plating, the medium was aspirated and fresh medium with or without retinol was placed in the wells. Seven days later, the cells were incubated with 20 μ g/ml of BrdUrd for 3 h and then fixed with 95% ethanol solution. The slides were stained by an indirect immunoperoxidase method with a mouse monoclonal antibody against BrdUrd. The labelling index, i.e., the percentage of positive nuclei, was determined by counting 500 or more cells.

The concentration of vitamin A in the FBS used was measured as that of retinol by high performance liquid chromatography [3].

Mean values of cell counts, CFE, and BrdUrd labelling index were analysed for statistical differences by Student's *t*-test with or without Welch's correction. In the degree of epithelial cell out-

growth, data of each group were compared using the Student's *t*-test for mean values and one-way ANOVA. All values of $p<0.05$ were considered to be significant.

Results

The effects of retinol on epithelial cell outgrowth from the explants of human peripheral lung tissue were examined by determining the number of grids occupied by the epithelial cells. Table 1 shows the ratio of the average grid number occupied by the cells in retinol-free (DMSO alone) and retinol-containing cultures (10^{-5}, 10^{-7}, 10^{-8} M) compared to that of the non-treated control cultures. In all experiments, the epithelial cell outgrowth in the retinol-containing cultures (10^{-8} M and 10^{-7} M) outnumbered that in the cultures with DMSO alone, although the difference was not significant statistically. The ratio at 10^{-5} M retinol was remarkably smaller than that of the DMSO and 10^{-7} M retinol group, though not statistically significant, suggesting that this concentration of retinol was inhibitory for the outgrowth of human distal airway epithelial cells.

Table 1 Degree of epithelial cell outgrowth with various concentrations of retinol. Each experiment was performed with explants of lung tissue obtained from different individuals. The degree of epithelial cell outgrowth is presented as the ratio of the average number of grids occupied by outgrown cells in dimethylsulphoxide (DMSO) or retinol-treated cultures to the average number of grids occupied by the non-treated control cultures. The values are derived from triplicate experiments that included nine explants in each group. There was no statistically significant difference between mean values of the DMSO group and the 10^{-8} M and 10^{-7} M retinol groups in Student's *t*-test. Also, values of each group, except the 10^{-5} M retinol group, were not significantly different in one-way ANOVA

Experiment	Retinol (M)			
	0	10^{-8}	10^{-7}	10^{-5}
1	0.3	1.2	1.7	–
2	2.8	4.1	6.1	–
3	1.4	4.2	3.4	–
4	0.9	–	1.4	0.4

In the retinol-containing cultures, the epithelial cells outgrown from the human lung explants were polygonal or cuboid, had a tightly compact arrangement, and a distinct cobblestone appearance (Fig. 1a), while those in the retinol-free cultures were spindle-shaped and flat and exhibited a rather loose arrangement (Fig. 1b).

We conducted preliminary studies on the CFE of epithelial cells harvested from the explant culture of human lung tissue, using fibroblastic feeder layers of four different cell types; Swiss 3T3 cells, NIH 3T3 cells, rat lung fibroblasts, and human lung fibroblasts. The Swiss 3T3 cells provided the optimal feeder effects for colony formation by the epithelial cells (data not shown). We therefore examined the effect of retinol on the CFE of human distal airway epithelial cells using the feeder layers of Swiss 3T3 mouse fibroblastic cells. Since the maintenance of fibroblastic feeder layers depends upon the serum concentration in the medium (data not shown), the cultures were incubated in medium containing 0%, 0.5%, 2% or 5% FBS. As shown in Table 2, in the cultures without retinol, the largest CFE was observed with 0.5% FBS. When 10^{-7} M or 10^{-5} M retinol was added to the cultures, the colony formation was reduced in a dose-dependent manner at each FBS concentration. This retinol-induced reduction in CFE was statistically significant with 0% and 0.5% FBS ($p<0.05$, respectively). Chemical analysis revealed that the FBS used in this study contained a very low concentration of retinol (10^{-9} M); thus it is reasonable to consider that the FBS did not contribute to any significant increase in the vitamin A concentration in the culture medium.

We examined the effect of retinol on epithelial cell growth in terms of cell population expansion on the plastic substratum. After 3 weeks of culture, primary epithelial cells were harvested from the primary cultures incubated with serum-free defined medium containing 10^{-7} M retinol. As shown in Figs. 2a, b, the number of

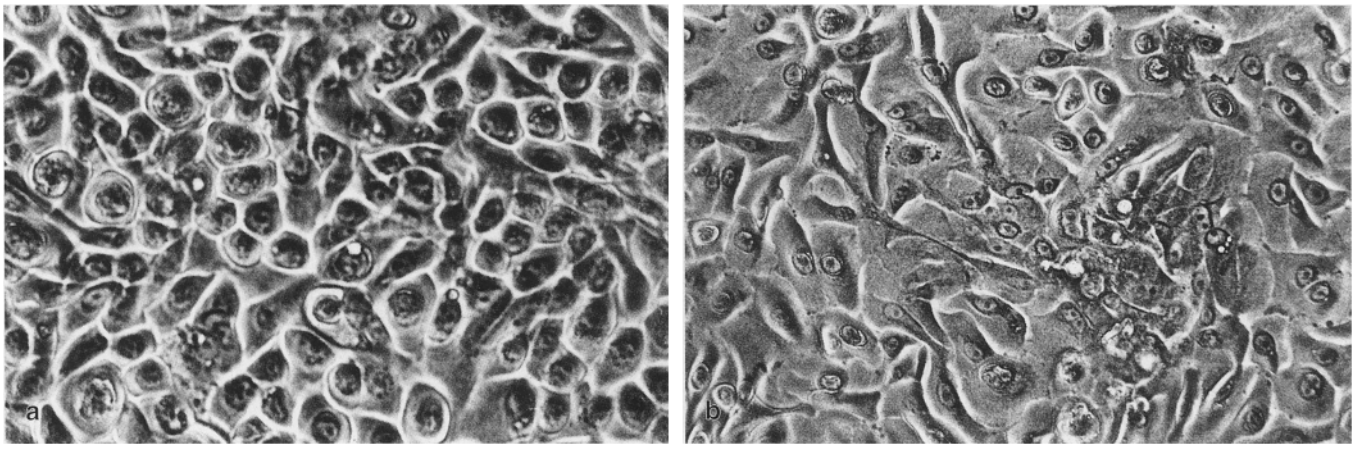

Fig. 1 Phase contrast photomicrograph of human distal airway epithelial cells in primary cultures at 3 weeks. Epithelial cells in the retinol-containing (10^{-7} M) primary culture are polygonal or cuboid (**a**), and the cells in the retinol-free culture are flattened or spindle-shaped (**b**). **a**, × 600; **b**, × 300

epithelial cells in the cultures without retinol was increased to three-fold the number of cells plated on day 7, regardless of the initial cell density. When retinol, at 10^{-7} M or 10^{-5} M, was added to the medium, the growth rate was significantly reduced in a dose-dependent manner (49% reduction with 10^{-7} M and 87% reduction with 10^{-5} M; $p<0.005$, respectively) (Fig. 2a). We investigated whether the growth inhibitory action of retinol was dependent on the cell density, since it was plausible that this effect of retinol was indirectly exerted through the mechanism of intercellular communication [17, 24]. As shown in Fig. 2b, at the lower cell density (1×10^4 cells per well), both the growth rate of epithelial cells and the growth-inhibitory effect of retinol were almost equal to those seen at the higher cell density (4×10^4 cells per well, Fig. 2a).

Table 2 Colony-forming efficiency (CFE) of epithelial cells on Swiss 3T3 fibroblastic feeders with various serum and retinol concentrations. Epithelial cells harvested from primary human lung tissue culture were plated onto Swiss 3T3 fibroblastic feeders, at a density of 2×10^3 cells per well; the cultures were maintained for 7 days. The CFE was determined as the percentage of the number of epithelial cell colonies against that of cells plated. The values are means ±SE of triplicate experiments. Figures in parentheses represent the ratio of CFE to that of the corresponding retinol-free culture.

FBS	Retinol (M)	CFE (%)	
0%	0	0.9±0.1	(1.00)
	10^{-7}	0.3±0.03**	(0.33)
	10^{-5}	0.1±0.05*	(0.11)
0.5%	0	4.2±0.3	(1.00)
	10^{-7}	2.5±0.4**	(0.59)
	10^{-5}	1.1±0.4*	(0.26)
2%	0	2.3±0.2	(1.00)
	10^{-7}	1.9±0.1	(0.83)
	10^{-5}	1.8±0.4	(0.78)
5%	0	1.6±0.1	(1.00)
	10^{-7}	1.3±0.1	(0.81)
	10^{-5}	1.0±0.1**	(0.63)

* $p<0.005$ compared to the value of the corresponding retinol-free cultures

** $p<0.05$ compared to the value of the corresponding retinol-free cultures

Fig. 2 Growth of human distal airway epithelial cells on plastic substratum and the effects of retinol and cell density. Human distal airway epithelial cells, harvested from primary cultures medium retinol at 3 weeks, were seeded, at 4×10^4 cells per well (**a**) or 1×10^4 cells per well (**b**) in 24-multiwell plates. The re-plated cells were cultured for 5 and 7 days, respectively, either without (●) or with retinol (△; 10^{-7} M, ▲; 10^{-5} M), and the number of cells was determined. Each point represents the mean value of triplicate experiments, and the vertical bar indicates SD. At 7 days, there was a significant reduction in cell number, not only in cultures with 10^{-7} M retinol at the lower cell density (**b**) but also in the cultures with 10^{-7} M and 10^{-5} M retinol at the higher cell density (**a**)

The effects of retinol and retinoic acid on the proliferation of human distal epithelial cells harvested from primary cultures grown with or without retinol were examined to determine whether the growth-inhibitory effects of retinol in these primary epithelial cells differed according to their differentiating status, and whether the presence or absence of retinol in the primary cultures altered the proliferative potential of the primary epithelial cells in the subculture. The results are shown in Table 3. While the epithelial cells derived from the retinol-containing primary cultures increased to 4.6- and 4.2-fold of the initial cell number in the non-treated and DMSO-treated subcultures, respectively, the cells from the retinol-free primary cultures increased only 2.8- and 2.6-fold, respectively. These increases were significantly different between cells from the retinol-containing and retinol-free cultures ($p<0.01$ and $p<0.005$, respectively). It was obvious that retinol inhibited proliferation in the secondary cultures of cells derived from both the retinol-containing and the retinol-free primary cultures. The growth-inhibitory effect of retinol was more prominent in cells from the retinol-free than in the cells from the retinol-containing primary cultures. There were statistically significant differences ($p<0.01$ with 10^{-10} M, $p<0.05$ with 10^{-8} M, $p<0.005$ with 10^{-6} M). Retinoic acid also had a similar inhibitory effect on cell growth, exerted in a clearly dose-dependent fashion. In contrast with the results for retinol, however, retinoic acid showed no difference in degree of growth inhibition between cells from the retinol-containing and cells from the retinol-free primary cultures.

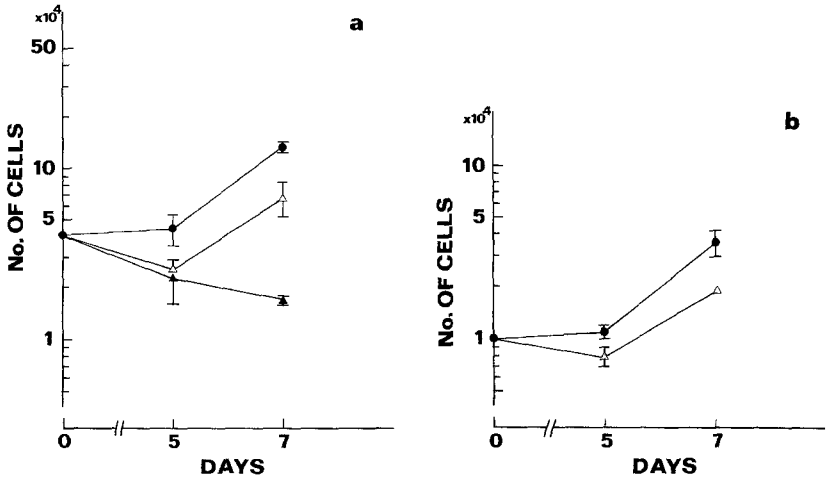

Table 3 Number of epithelial cells in secondary cultures treated with various concentrations of retinol or retinoic acid. Epithelial cells were harvested from retinol-containing and retinol-free primary cultures. Pooled cells in each group were plated, at 4×10^4 cells per well, and were further cultured for 7 days under the conditions indicated. The values are means ±SE of triplicate experiments. Figures in parentheses indicate the ratio of the cell number to those of the non-treated control.

Conditions of secondary culture	Cell number ($\times 10^4$)	
	Cells from retinol-containing primary culture	Cells from retinol-free primary culture
No treatment	18.3±1.2 (1.0)	11.3±0.9 (1.0)
DMSO alone	16.9±0.4 (0.92)	10.4±0.9 (0.92)
Retinol		
10^{-10} M	9.8±0.7** (0.58)	2.7±0.06**** (0.26)
10^{-8} M	8.2±0.8* (0.49)	2.9±0.06**** (0.28)
10^{-6} M	7.0±0.3* (0.41)	1.6±0.06*** (0.15)
Retinoic acid		
10^{-10} M	9.4±1.0* (0.56)	7.7±0.5 (0.75)
10^{-8} M	5.1±0.3* (0.30)	3.7±0.06**** (0.36)
10^{-6} M	1.5±0.2* (0.09)	0.3±0.06*** (0.03)

* $p<0.001$ compared with DMSO group
** $p<0.005$ compared with DMSO group
*** $p<0.01$ compared with DMSO group
**** $p<0.05$ compared with DMSO group

Table 4 Bromodeoxyuridine (BrdUrd) labelling index of epithelial cells in secondary cultures with and without retinol treatment. The secondary cultures were incubated at 7 days for 3 h with BrdUrd, the cells were then fixed with 95% ethanol, and BrdUrd-positive cells were visualized by the immunoperoxidase method. The values are the means ±SE of triplicate experiments.

Conditions of secondary culture	Labelling index (%)	
	Cells from retinol-containing primary culture	Cells from retinol free primary culture
DMSO alone	32.0±0.9	21.5±0.9
Retinol		
10^{-7} M	25.5±0.6*	17.5±1.0**
10^{-5} M	0	0

* $p<0.01$ compared with DMSO group
** $p<0.05$ compared with DMSO group

Table 5 Colony-forming efficiency of epithelial cells derived from primary cultures with and without retinol. Epithelial cells were harvested from retinol-containing or retinol-free primary cultures and pooled cells in each group were seeded, at 4×10^3 cells per well, on Swiss 3T3 fibroblastic feeders. The cultures were maintained for 7 days with DMSO alone or with 10^{-7} M retinol. They were stained by the immunoperoxidase method with an anti-keratin antibody, and the number of keratin-positive colonies of 30 or more cells was counted. The values are means ±SE of triplicate experiments.

Conditions of secondary culture	Colony-forming efficiency (%)	
	Cells from retinol-containing culture	Cells from retinol-free primary culture
DMSO alone	2.1±0.2*	1.3±0.06*
Retinol		
10^{-7} M	1.0±0.02**	0.3±0.03***

* $p<0.05$ between the two values
** $p<0.05$ compared with DMSO group
*** $p<0.001$ compared with DMSO group

The replication of epithelial cells from both the retinol-free and retinol-containing primary cultures, measured in terms of the BrdUrd labelling index was significantly inhibited by 10^{-7} M retinol ($p<0.05$ and $p<0.01$, respectively) and was completely abolished by 10^{-5} M retinol (Table 4).

The effect of retinol at 10^{-7} M on the CFE of epithelial cells was compared in cells derived from the retinol-containing and retinol-free primary cultures. As shown in Table 5, the CFE was significantly decreased by treatment with 10^{-7} M retinol in both groups. The rate of growth inhibition appeared to be greater in the cells from

Table 6 Number of human distal airway epithelial cells after serial subcultivation with and without retinoic acid. Epithelial cells harvested from retinol-containing primary cultures were pooled and plated, at 3×10^4 cells per well, in multi-well plates. The cells were cultured for 7 days with or without 10^{-8}M retinoic acid (RA). Cells were then harvested and the cell number was determined. The cells in each group were pooled and replated again, at 3×10^4 cells per well. Seven days later, the number of cells was determined. The values are means ±SE of triplicate experiments.

Treatment of subculture	Cell number ($\times 10^4$)	
	Yield of cells in second subculture	Yield of cells in third subculture
DMSO alone	7.2±0.3	6.1±0.1
RA (10^{-8}M)	4.9±0.5*	16.1±0.4**

* $p<0.05$ compared with DMSO group
** $p<0.001$ compared with DMSO group

the retinol-free than in cells from the retinol-containing culture. There was statistically significant differences between cells derived from the retinol-containing and retinol-free primary cultures ($p<0.01$ without retinol, $p<0.005$ with 10^{-7}M retinol). The CFE in the cultures treated with DMSO alone was greater in the cells from the retinol-containing than in cells from the retinol-free cultures. When the results of the studies of cell population, BrdUrd labelling index, and CFE were combined, it was apparent that cells derived from the primary culture treated with 10^{-7}M retinol had greater proliferative potential than cells from the primary culture without retinol.

To validate the above observation, we carried out the following experiment with retinoic acid. Primary epithelial cells grown in cultures with 10^{-7}M retinol were subcultured with or without retinoic acid. Cells in the second cultures in each group were pooled and replated, and third subcultures were carried out for 7 days. As shown in Table 6, retinoic acid inhibited epithelial cell growth in the second subculture. It is of interest that the epithelial cells from the second subculture with retinoic acid exhibited a higher yield of cells in the third subculture than cells from the second subculture not incubated with retinoic acid.

Discussion

In most instances, vitamin A inhibited the growth of human distal airway epithelial cells in culture. The inhibition of growth by retinol and retinoic acid seemed to be dose-dependent at the doses we examined, although the concentrations of these agents were much higher than those in the normal physiological state. Retinol enhanced the outgrowth of epithelial cells from the explants, the explants being composed of various types of cells and abundant extracellular substances. It has been reported that fibroblasts and macrophages produce factors that are closely involved with the proliferation of respiratory epithelial cells [11, 27]. Studies using organ cultures of tracheal fragments have disclosed vitamin A analogues that exhibit growth-promoting action in respiratory epithelial cells [9]. In the presence of an extracellular matrix substratum, the growth of respiratory epithelial cells was promoted by retinoic acid [16, 19, 29]. It seems likely that some kinds of extracellular matrix and/or cells other than epithelial cells, in combination with vitamin A, supported the proliferation of the epithelial cells in the primary culture of human lung explants.

We also examined the effects of vitamin A on proliferation of human distal airway epithelial cells, isolated from the primary culture, namely, from the explants of lung tissue, and plated onto feeder layers or plastic substratum. By employing these subculture methods, we were thus able to examine and quantitate the direct effects of vitamin A. We found that retinol inhibited not only the colony formation of these cells on feeders but also the population increase rate on plastic substratum. DNA synthesizing activity was also inhibited by treatment with retinol. We also found that the proliferative activity of primary culture-derived cells incubated in the presence of retinol was greater than that of cells derived from the control primary culture in subcultures treated with various concentrations of retinol or retinoic acid. Retinoic acid exhibits diverse effects on the proliferation of different types of respiratory epithelial cells [2, 16]. It is plausible that the tolerance of cells derived from the retinol-treated culture to the growth-inhibitory effect of vitamin A was induced through the selection of resistant cells. It is also possible that retinol stimulated the proliferation of a subpopulation of cells with greater growth potential, and that then the expansion of such a cell population ensued as a result of the treatment with retinol. The results of the serial subculture study seemed to be consistent with both of these explanations, since the population increase was enhanced in the third culture of cells derived from the second culture in which the proliferation of cells in total appeared to have been suppressed by treatment with retinoic acid.

Vitamin A controls the differentiation of the respiratory epithelium [7, 15]. Whereas the epithelial cells of the human distal airway differentiate into epidermoid cells in retinol-free, as well as in serum-free, culture [8], retinol or retinoic acid promotes the differentiation of the cells into ciliated cells and nonciliated secretory cells. Hence, it is possible that the growth potential of epithelial cells might be altered by vitamin A deficiency, producing an alteration in differentiation. For example, if epidermoid metaplasia involves the terminal differentiation of virtually all cells, the cells would completely lose the proliferation and cell division in capacity.

In summary, this study showed that vitamin A inhibited the proliferation of human distal airway epithelial cells isolated from a primary explant culture and subcultured on feeder layers or a plastic substratum. In the primary culture that contained lung tissues, in contrast, the proliferation of these cells was enhanced by retinol. These results indicate that vitamin A may exert different ef-

fects on cell replication depending upon the environment of cells, in particular whether they are isolated from or associated with other types of cells and the extracellular matrix. Our results also suggest that the human distal airway epithelial cells contained subpopulations of cells that showed different responses to vitamin A; some cell fractions were resistant to the growth-inhibitory effect of vitamin A and were even stimulated to proliferate. While histology of their explants was normal, the tissues were taken from lungs resected for cancer. It is thus possible that the status of the starting material and vitamin A status of the patients may have affected the subsequent responsiveness of the cultured tissues to exogenous retinol. In any case, the results of this study provide important information on the development and potentially, the control of human lung cancer, especially of the peripheral type.

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