GROWTH AND DIFFERENTIATION OF CONDUCTING AIRWAY EPITHELIAL CELLS IN CULTURE

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Running Title: Airway Epithelial Cell Differentiation

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ABSTRACT

The development of routine techniques for the isolation and in vitro maintenance of conducting airway epithelial cells in a differentiated state provides an ideal model to study the factors involved in the regulation of the expression of mucociliary differentiation. Several key factors and conditions have been identified. These factors and conditions include the use of biphasic culture technique to achieve mucociliary differentiation and the use of such stimulators, the thickness of collagen gel substratum, the calcium level, and vitamin A, and such inhibitors, the growth factors EGF and insulin, and steroid hormones, for mucous cell differentiation. Using the defined culture medium, the life cycle of the mucous cell population in vitro was investigated. It was demonstrated that the majority of the mucous cell population in primary cultures is not involved in DNA replication. However, the mucous cell type is capable of self-renewal in culture and this reproduction is vitamin A dependent. Furthermore, differentiation from non-mucous cell type to mucous cell type can be demonstrated by adding back a positive regulator such as vitamin A to the "starved" culture. Cell kinetics data suggest that vitamin A-dependent mucous cell differentiation in culture is a DNA replication-independent process and the process is inhibited by TGF-β1.

1. INTRODUCTION

The epithelial lining of the conducting airways provides a barrier and first line of defense to inhaled particles and toxic gases. Two properties inside the epithelium are essential elements of the defense mechanism. One of them is the production of mucus, which plays a major role in trapping the inhaled particules before they reach the epithelium. The other property is the ciliary beating that removes these particles trapped in mucus to the larynx where they are then swallowed to stomach. The cell type responsible for the ciliary beating is the ciliated cell type. This cell type is regarded as a terminal cell type and is not involved in the renewal of airway epithelium (1). The ciliated cells have proven to be relatively stable under both the in vivo hostile environment and the in vitro culture condition. The cell type responsible for the mucus production in the surface airway epithelium is the mucous cell type. Other cell types located in the submucosal gland region also contribute to mucus production. We focus our study on the differentiation of mucous cells of surface airway epithelium. Mucin, the major component responsible for the vascoelastic properties of mucus (2), is produced by these cells. Airway injury or disease often results in the compromise of this protective function by the over-production and/or alteration in the content of airway mucin. The nature of this alteration is largely unknown.

To understand the nature of mucus production, a number of in vitro models, including the organ culture and the cell culture systems, have been developed (3-6). In this communication, we focus on the cell culture model that permits epithelial cells to express ciliogenesis and mucous glycoprotein

synthesis. We use this model to study the regulation of the mucin synthesis by various micro-environmental nutrients.

2. CELL CULTURE MODEL OF AIRWAY EPITHELIAL CELLS

2a. The Development of Serum-Free Hormone-Supplemented Medium

There are numerous ways that airway epithelial cells can be cultivated in vitro over long periods. Techniques have been developed that allow us to obtain viable epithelial cells from different airway regions (7,8). These epithelial cells, in most cases, are cultivated in a serum-free medium supplemented with a mixture of hormones and growth factors (4-6). In a serum-supplemented medium, we observed limited cell proliferation and short life span, except in a few cases when airway epithelial cells were cultivated in the presence of low amount of serum. Lechner and his colleagues were first to show that this limited proliferation is due to the presence of TGF-β in the serum (9). TGF-β is a potent growth inhibitor for airway epithelial cells. This result is consistent with the concept made many years ago by Sato and his colleagues (10) that cell growth, especially the epithelial cell type, is regulated by hormones and various growth factors. Based on the response of primary airway epithelial cells to various growth factors, we have systematically developed a serum-free hormone-supplemented medium for growth of various airway epithelial cells derived from different ages and airway segments, including those from fetus and distal airways. The hormonal growth supplements used in our study are: insulin, transferrin, epidermal growth factor, hydrocortisone, cholera toxin, bovine hypothalamus extract, and retinol. Except for retinol, the

physiological significance of these supplements in vivo are still unknown. Using this defined medium, up to 5 passages and 25-30 population doublings have been demonstrated in airway epithelial cells obtained from an adult human (6).

In contrast with the development of cell proliferation condition, the development of a culture condition for ciliogenesis and mucous cell differentiation has been more troublesome. The difficulty in developing an ideal culture condition for airway mucociliary cell differentiation is in part because of the complexity of the regulation of cell differentiation in which the life cycle of various airway epithelial cell types is still unresolved. For mucous glycoprotein synthesis, the complexity includes the regulation at the transcriptional and translational levels, the glycosylational steps, and the processes involved in storage and secretion. We are also hindered by our limited knowledge of the biochemical and genetic structures of large molecular weight mucous glycoproteins. The complex structures and the diverse types of mucins are well recognized. In order to elucidate the airway mucin synthesis in culture, previous studies had to rely characteristics of several biochemical properties of mucin, such as the amino acid and carbohydrate compositions, the molecular size and enzymatic characterization, and the presence of O-glycosidic bonds in the isolated molecules (2, 11-13). Since few biochemical or genetic probes are available for quantitating mucin synthesis, these characteristics are initially essential in order to ascertain the mucin-like synthesis. However, these characteristics are not practical in the routine quantitation of mucin synthesis and mucous cell population in culture. Several monoclonal antibodies which are useful in the identification of mucous cell population and the quantitation of mucin

synthesis have recently been developed (14,15). These antibodies are specific to airway mucin and secretory mucus granules. Only recently, has mucous cell differentiation been studied at the genetic level. Several putative apomucin cDNA clones have been used as probes for measuring the mRNA level of a specific apomucin gene (17-21).

2b. Evidence of Mucociliary Cell Differentiation in Culture.

The primary culture system of hamster tracheal epithelial cells is the first in vitro demonstration of mucous cell differentiation (11-13) and ciliogenesis (12,22). The culture system was identical to the serum-free hormone-supplemented medium for cell growth except the use of a collagen gel substratum. Evidence of mucous cell differentiation in the hamster culture was based on the biochemical analyses of mucin-like synthesis, such as the molecular size and the enzymatic digestion, the compositional analyses of the secretion, and the presence of mucous granules in cultured cells. Furthermore, the quantitative kinetics and the time-course studies suggest that the mucous cell population in the hamster culture is derived from the undifferentiated cell type. Evidence of in vitro mucous cell differentiation has now been extended to cultures derived from monkey, human (6), rabbit (23) and guinea pig (24) airway tissues. Recently, the development of mucin-specific monoclonal antibodies allow us to use both the ELISA (25) and immunohistochemical methods (6) to quantitate the mucin production and mucous cell population, respectively, in culture. Data obtained from these two methods are further supported by the morphological characterization. Using these two quantitative methods, several in vitro parameters affecting

the mucous cell differentiation have been investigated in human and monkey cultures.

The other advance in the in vitro culturing airway epithelial cells is the development of biphasic culture system in which epithelial cells are maintained in air-liquid medium interface (24, 28-30). This new culture system reflects the in vivo situation and allows further cell differentiation. We have observed mucociliary differentiation of cultured human and monkey airway epithelial cells that are maintained in the biphasic culture under the basal feeding but not under the traditional immersed feeding. A similar observation has been demonstrated by other investigators (31,32). This development supports the feasibility to study the mechanisms and factors that are involved in the regulation of mucociliary differentiation in vitro. However, we found that it is still difficult at this moment to carry out the study related to ciliogenesis because lack of method for assessing ciliogenesis quantitatively. For mucous cell differentiation, both ELISA and immunocytochemistry methods have been developed for quantitating mucous cell population in culture.

3. EFFECTS OF CULTURE CONDITIONS ON MUCOUS CELL DIFFERENTIATION

3a. Effects of Collagen Substratum

The collagenous substratum has been used in various cell culture systems, including the respiratory epithelial cells, to promote cell proliferation and differentiation. Lechner and his colleagues have developed a thin substratum with a mixture of type I collagen, bovine serum albumin,

and fibronectin to enhance human bronchial epithelial cell attachment and proliferation (5). However, the effect of this type of culture system on airway epithelial cell differentiation was not studied. We used a collagen gel substratum to enhance cell proliferation of both hamster (12,13,22) and mouse (25) tracheal epithelial cells. It was the first quantitative demonstration that new cilia and mucous granules were formed in the hamster cultures that are maintained on the collagen gel substratum. These results prompt the initiation of several studies in the differentiation of cultured airway epithelial cells derived from rabbit, guinea pig, human, and non-human primate tissues. All of these studies draw a similar conclusion that, at least, the expression of the airway mucous cell differentiated function is enhanced by a collagenous substratum. However, the presence of a collagenous membrane is still insufficient to promote new ciliogeneous in vitro except in a few cases, such as the primary hamster and guinea pig tracheal epithelial cultures.

Recently, we observed some differences when we compared the differentiated function of mucous cells plated on the thin collagen-coated surface and the thick collagen gel-coated surface. To further understand the nature of this substratum effect, both human and primate cells were plated on a collagen gel (CG) surface with different thicknesses. Using both the biochemical and immunological methods of mucin synthesis, we observed that the activity of mucous cell differentiation in culture is dependent on the CG thickness. At CG less than 0.5 ml per 35 mm tissue culture dishes, mucous cell function is significantly reduced. These results suggest that the optimal mucous cell differentiation in culture requires a collagen gel substratum at least 1 mm thick (26).

3b. Effects of the Calcium Levels

The importance of calcium in cell proliferation and cell differentiation has been well recognized. On a plastic surface, a low calcium (<0.1 mM) medium prohibits epithelial cells from expressing terminal differentiation. However, the effects of calcium on airway epithelial cells grown on a CG substratum have not been investigated. We prepared our own F12 medium in which the level of calcium could be manipulated. Using this homemade F12 medium, we investigated the effects of the calcium level on mucous cell differentiation. We observed that the expression of mucous cell function is dependent on the concentration of calcium in the medium. These studies demonstrate that > 0.3 mM calcium in culture medium is needed to achieve mucous cell differentiation (27).

3c. Effects of Retinoid

The important role of vitamin A and its derivatives (retinoids) in maintaining the homeostasis of airway epithelium, especially the mucous cell differentiation, has been well recognized. However, the nature of this effect has not been elucidated. The development of the serum-free hormone-supplemented medium for airway epithelial cell culture provides an opportunity to elucidate the role of retinoid on the regulation of mucous cell differentiation. When vitamin A was removed from this serum-free culture medium, the expression of mucous cell function, specifically the mucin synthesis, was greatly depressed in human airway cell cultures (Fig. 1). This result was further supported by the EM and by the immunological studies. This phenomenon was not observed when other growth supplements were

removed from the culture medium. Similar observations have been described in primary epithelial cultures of hamster and monkey airways. These results are consistent with studies performed both in vivo and in vitro organ culture system, suggesting that vitamin A plays the stimulatory role in the regulation of mucous cell differentiation.

3d. Effects of Other Steroid Hormones and Growth Factors

The removal of several growth factors, such as insulin, and EGF, from the culture medium caused a severe reduction in cell number but had a stimulatory effect on the mucin synthesis activity on a per cell basis (Fig. 1). These results suggest that these growth promoting factors are inhibitors of mucous cell differentiation. In the case of the glucocorticoid steroid hormone, the cell number slightly decreased in the minus hydrocortisone culture condition. However, the mucin synthesis was greatly stimulated in the absence of this steroid hormone (Fig. 1). To further understand the effect of steroids, we investigated the effects of the thyroxine hormone (T_3) since this chemical has a similar nuclear receptor as the one for the glucocorticoid steroid. As shown in Table I, T_3 has an inhibitory effect on the expression of mucin synthesis. This inhibition is less than HC and Dex. Furthermore, neither HC nor T_3 can replace the vitamin A role in stimulating mucous cell function. These studies suggest that steroid hormones and T_3 are inhibitors of mucous cell differentiation. Further studies are needed to elucidate the regulation.

4. PLASTICITY OF MUCOUS CELL DIFFERENTIATION IN CULTURE

4a. Plasticity of Vitamin A-Mediated Mucous Cell Differentiation.

One of the benefits of using an in vitro approach is to be able to manipulate the level of the growth supplement of interest in the culture medium. By depleting or adding this growth supplement to the culture environment, the response of cells to this growth supplement can be analyzed in detail. Using this type of approach, we studied the plasticity of in vitro mucous cell differentiation in response to vitamin A. We showed that the expression of mucous cell function, measured as the mucin production, in the "vitamin A starved" cultures can be re-initiated with the addition of vitamin A (Fig. 2). However, the activity of this re-initiation phenomenon decreased with culture time. This result suggests that the response of cultured cells to vitamin A is time dependent and the plasticity of the response is quite unstable in culture. To further understand the nature of this effect, we conducted the following experiments to determine the origin of mucous cell population in culture and the response of this population to the vitamin A treatment.

4b. Origin of the Mucous Cell Population in Cultures

Mucous cells are not a terminal cell type. In a normal, uninjured state, in vivo mucous cells are capable of incorporating ^{3}H-thymidine into their DNA (1). It has been suggested that mucous cells serve as a progenitor cell type responsible for the regeneration of airway epithelium in response to injury (1). This role is less clear in the in vitro culture since the progenitor-precursor relationship is not as apparent as that existing in vivo.

A 4-day old vitamin A-treated culture of human tracheobronchial epithelial cells was labeled with ^{3}H-thymidine for 2 hrs. After the labeling, the culture was chased with cold thymidine and the cultured cells were harvested at different times. We observed that 25% of the cell population incorporated the ^{3}H-thymidine during the 2 hr labeling (Table II). Among the labeled cells, less than 25% stained with mucin-specific antibody (designated as M+/N+ in Table II). After 48 hr chase, the labeling index increased to 70% of the total cell population. Among the radioactively labeled cells, 30% stained with the mucin antibody. These results suggest that half of the mucous cell population was from the replicative pool of either mucous or non-mucous cell populations. However, half of the mucous cells in this study were not derived from the replication pool. In the case of non-mucous cells in culture, less than 20% of them were not derived from the replication pool.

In contrast to the above results, the labeling index in the absence of vitamin A was 25% and none of these labeled cells were stained by the mucin-specific antibody despite 20% of non-labeled cells stained by this antibody were observed in this culture (Table II). After 48 hr chase, the labeling index was 75%, but none of the labeling was incorporated into the cells stained with the mucin-specific antibody. These results suggest that vitamin A has two significant effects on the life cycle of mucous cell population in culture. One is that vitamin A is required for the replication pool of mucous cell population. Second, the conversion from non-mucous cell type to mucous cell type is vitamin A dependent.

4c. Role of DNA Replication in Vitamin A-Induced Mucous Cell Differentiation

The above experiments established that mucous cells can be derived from the replicating pool of non-mucous cells. However, it is not clear whether DNA replication is a necessary step for this conversion. To address this question, we repeated the labeling experiment using a primary culture which was starved for vitamin A. We supplemented this culture with vitamin A at a later time (such as at day 8) when DNA synthesis was low. As shown in the Table III, the labeling index of this culture was 6% and none of the labeled cells were of the mucous cell type. Furthermore, the mucous cell type was about 5% of the total cell population. After 24- and 48-hr chase, the labeled cells increased to the 30% level and again, none of them were of the mucous cell type. When vitamin A was added back to the culture, the labeling index did not change immediately. However, the mucous cell population increased to 19% and 30% respectively, at 24- and 48-hr chase. The mucous cells that were radioactively labeled were less than 5%. These results suggest that the majority of mucous cell population appeared in the culture after the vitamin A treatment was not derived from the replication pool. It is suggested that the conversion from non-mucous cell type to the mucous cell type by the vitamin A treatment does not need to include the DNA replication step.

Recently, we have begun to examine the nature of vitamin A-dependent mucous cell differentiation. Data not present in this communication include the study of TGF-β1 inhibition. We have observed that this differentiation step is inhibited by TGF-β1 and the inhibition is a TGF-β1 dose-dependent phenomenon.

5. CONCLUSION

The sucessful development of culture condition for conducting airway epithelial cells is based on the concept developed by Sato and his colleagues, that cell growth is regulated by hormones and growth factors. Several serum-free hormone-supplemented media have been developed to optimize the epithelial cell growth. We used the defined culture system to further optimize the condition for airway mucociliary cell differentiation. These studies identified the stimulators and inhibitors involved in the regulation of mucous cell differentiation. The physiological significance of these findings remain to be elucidated. Through the defined culture condition, it is now possible to investigate the mechanisms involved in the regulation of airway epithelial cell differentiation. Vitamin A is an important factor involved in the in vitro regulation of mucous cell differentiation. By manipulating the level and the time of vitamin A in this defined culture condition, we observed a complicated scheme in generating the mucous cell population. Some of the mucous cells are self-replicating, and some of them are derived from non-replicating, non-mucous cell type. It appears that DNA replication is not required for the vitamin A-dependent conversion from certain non-mucouc cells to mucous cells. The relationship between these different schemes needs to be explored further.

ACKNOWLEDGEMENT

The project was supported in part by grants from NIH (HL35635, HL54678, ES06230) and American Cancer Society.

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Table I: Effects of steroid hormone and T_3 on mucin production in primary cultures of human tracheobronchial epithelial cells.

Culture condition*	Mucin production	
	- Vitamin A	+ Vitamin A
	(ng/10^6 cells/24 hr)**	
-	8.25 ± 1.21	31.20 ± 2.56
+ HC	4.05 ± 0.52	19.81 ± 0.98
+ Dex	4.13 ± 1.21	18.89 ± 1.57
+ T_3	5.50 ± 0.87	22.88 ± 1.89
+ HC, + T_3	4.15 ± 0.53	16.71 ± 1.56

* Primary cultures of human tracheobronchial epithelial cells were grown on a collagen gel substratum in a F12 medium supplemented with 1.8 mM $CaCl_2$, and insulin, transferrin, cholera toxin, EGF, and bovine hypothalamus extract (26). At day 2, vitamin A (retinol, 1 μM), hydrocortisone (HC, 1 μM), dexamethasone (Dex, 0.1 μM), and T_3 (0.03 μM) were added as indicated. Media were changed every other day. Media were collected at day 9-10 and used for mucin-ELISA quantitation (16). Cell number in each dish was determined at day 10 and was used as the cell density in the calculation.

** Mucin ELISA method was carried out as described in the previous publication (16) using the purified mucin of human airway sputum. The reference mucin was expressed as the amount of protein based on the amino acid compositional analysis. Data were obtained from 4 dishes of each culture condition and expressed as means and s.d..

Table II: Effects of vitamin A on the life cycle of primary cultures of human tracheobronchial epithelial cells.

	Cell type distribution*				
Culture condition**	M+/N+	M+/N-	M-/N+	M-/N-	Total
	(Cell number counted)				
+ Vitamin A, day 4	22	135	66	134	357
(%)	(6.2)	(37.8)	(18.5)	(37.5)	(100)
Chase 2 days	55	53	125	23	256
(%)	(21.5)	(20.7)	(48.8)	(9.0)	(100)
- Vitamin A, day 4	0	70	87	193	350
(%)	(0)	(20.0)	(24.8)	(55.1)	(100)
Chase 2 days	0	48	303	54	405
(%)	(0)	(11.9)	(74.8)	(13.3)	(100)

* Cell type distrubution was examined under a light microscope with a 40X objective len. Dissociated cultured cells were cytospun on a glass slide and fixed in methanol:acetone (1:1). These slides were stained with the mucin-specific antibody by the Vectastain ABC peroxidase system as described before (14). After the immuno-staining, these slides were counter-stained with toludine blue and then coated with the L-4 emulsion (Polyscience, Inc.). Autoradiograph was carried out at 4° C for 2 weeks under the dark. Cells stained with the mucin-specific antibody are brown and are designated as M+. In contrast, cells not stained by this antibody are blue and are designated as M-. Cell nucleus labeled by ^{3}H-thymidine is shown as N+, otherwise, it is shown as N-, indicating no incorporation.

** Primary human tracheobronchial epithelial cells were maintained on a collagen gel substratum as described in Table I. At day 2, vitamin A was added, and at day 4 cultures were pulse labelled with ^{3}H-thymidine (5 μCi/ml) for 1 hr, than chased with the fresh medium for 2 days.

Table III: Effects of the vitamin A treatment on the life cycle of primary human tracheobronchial epithelial cells in culture.

Culture condition**	Chase (hrs)	Cell type distribution* M+/N+	M+/N-	M-/N+	M-/N-	Total
		(Cell number counted)				
Minus vitamin A	0	0	15	18	253	286
(%)		(0)	(5.2)	(6.3)	(88.5)	(100)
	24	0	4	136	298	438
(%)		(0)	(0.9)	(31.1)	(68.0)	(100)
	48	0	4	129	314	447
(%)		(0)	(0.9)	(28.9)	(70.2)	(100)
Add vitamin A	0	2	10	11	126	149
at day 8 (%)		(1.3)	(6.7)	(7.4)	(84.6)	(100)
	24	4	38	51	124	217
(%)		(1.8)	(17.5)	(23.5)	(57.1)	(100)
	48	12	76	86	120	294
(%)		(4.1)	(25.9)	(29.3)	(40.8)	(100)

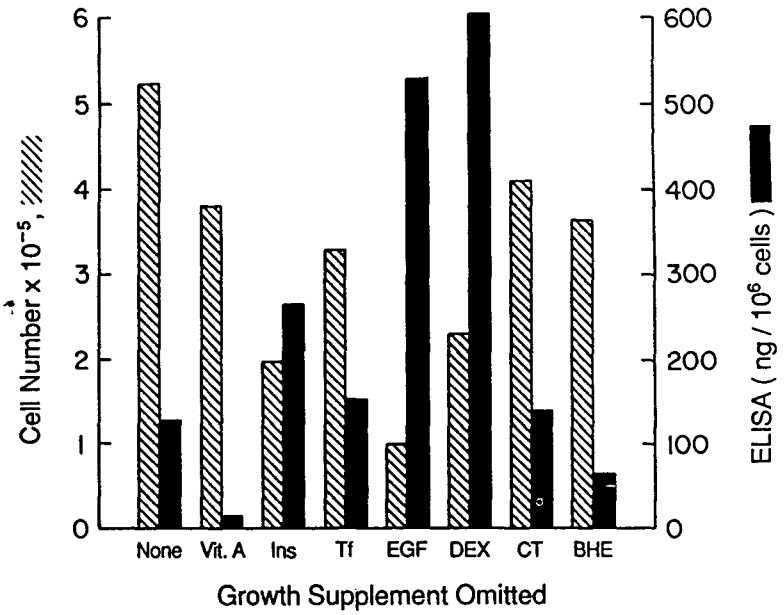
* Cell type identification was carried out as described in Table II.

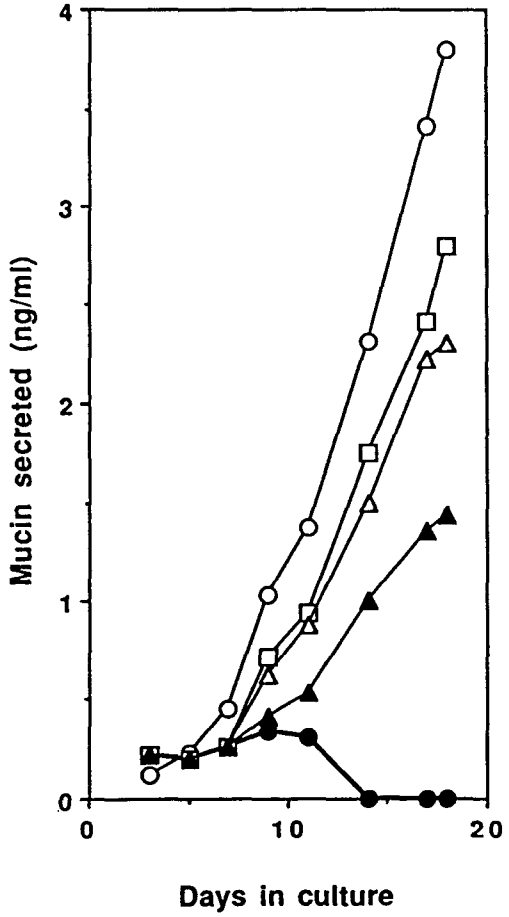
** Human primary tracheobronchial epithelial cells were maintained on a collagen gel substratum in the serum-free medium without vitamin A. At day 8, half of the culture were treated with vitamin A (retinol, 1 μM). At the same time, these cultures were treated with ^{3}H-thymidine for 1 hr (0 hr). The cultures were than chased with the fresh medium for 24- and 48-hr.

FIGURE LEGENDS

Fig. 1: Effects of hormonal/growth factor supplements on cell growth and mucin secretion in primary cultures of human tracheobronchial epithelial cells. Protease dissosiated human tracheobronchial epithelial cells were plated on collagen gel at 5 x 10^4 cells/35 mm dish in the serum-free medium supplemented with all or any 6 of the following 7 growth supplements: insulin (Ins), transferrin (Tf), epidermal growth factor (EGF), dexamethasone (dex), cholera toxin (CT), bovine hypothalamus extract (BHE), and vitamin A (retinol). At day 8, media were changed and collected 24 hrs later. The amount of the mucin in the culture media was determined by an ELISA method using the purified sputum mucin as a standard and expressed as ng of protein per million cells (filed bars). Cell number in each dish was determined at the same time (shade bars). Data were averages from triplicate dishes.

Fig. 2: Time course effects of vitamin A treatment on primary cultures starved for vitamin A. Human tracheobronchial epithelial cells were plated on a collagen gel substratum in a vitamin A-free medium (o). At day 1 (o), 3 (•), 4 (Δ), and 7 (Δ), retinol (1 μM) was added. Media were collected every day and the mucin content was determined by an ELISA method (16).


Cell Number x 10⁻⁵; ////
ELISA (ng / 10⁶ cells)
0
1
2
3
4
5
6
100
200
300
400
500
600
None
Vit. A
Ins
Tf
EGF
DEX
CT
BHE
Growth Supplement Omitted


Mucin secreted (ng/ml)
0
1
2
3
4
0
10
20
Days in culture