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Effect of Substratum and Retinoids upon the Mucosecretory Differentiation of Airway Epithelial Cells *In Vitro*

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The lining of the trachea consists of a pseudostratified, mucociliary epithelium that under a variety of conditions, such as vitamin A deficiency, toxic and mechanical injury, becomes a stratified squamous epithelium. Several *in vitro* cell culture models have been established to study the regulation of the mucosecretory phenotype. Such studies have indicated that the mucosecretory phenotype in tracheal epithelial cells can be modulated by substratum and the presence of retinoids. Cells grown on a collagen type I gel matrix in the absence of retinoids undergo stratification and squamous cell differentiation. Cells grown on a collagen gel matrix in the presence of retinoids express a mucosecretory phenotype. As in the normal tracheal epithelium, these cultures contain columnar, polarized cells that exhibit apical tight junctions and secretory granules. Biochemical analysis of radiolabeled glycoconjugates released into the medium indicate the synthesis of mucinlike glycoproteins. Retinoids appear to determine whether tracheal epithelial cells become committed to a pathway of squamous differentiation or to a mucosecretory pathway of differentiation. The collagen gel matrix appears not to determine the commitment of the pathway of differentiation but allows the expression of the secretory phenotype in retinoic acid-treated cultures. The mechanisms by which retinoids and substratum modulate differentiation in tracheal epithelial cells is still poorly understood. It is clear that differentiation into squamous or mucous cells requires the activation and suppression of different genes. In the case of retinoids, the alterations in gene activity may be mediated by the nuclear retinoic acid receptor. In summary, in tracheal epithelial cells the substratum and extracellular matrix in conjunction with hormonal factors such as retinoids determine the ultimate function of these cells.

Introduction

The epithelium of the mammalian upper respiratory tract is an attractive tissue in which to study differentiation for a variety of reasons. The tissue has a moderate degree of complexity, with a few major differentiated cell types. The tissue is easily accessible, especially for the instillation of substances that may alter differentiation. Recently, the tissue has become more accessible in an experimental sense as methods have been developed to study proliferation, differentiation, and differentiated functions, in *in vitro* systems. Finally, the tissue expresses interesting and clinically important differentiation-related pathology in the form of squamous metaplasia that can occur as the result of a variety of insults to the epithelium. The present chapter will review recent advances in the study of airway cell differentiation *in vitro* with emphasis upon the effects that retinoids and substratum have in modulating this process.

Functional Definition of Airway Epithelial Cell Differentiation

Several cell types have been observed within the pseudostratified epithelium of the upper airway (*1–3*). Basal cells are in contact with the basement membrane but do not reach the lumenal surface of the epithelium. These cells have a high nuclear-to-cytoplasmic volume ratio and have, at least until recently, been thought to be the stem cell of the epithelium, thus giving rise to the other more differentiated cell types. Mucosecretory cells and ciliated

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cells are the major differentiated cell types present. These are columnar in shape with their basolateral surfaces in contact with the basement membrane while their apical surfaces are exposed on the lumenal surface of the epithelium. The epithelium is termed pseudostratified because of an apparent stratification of a lower layer of basal cells and an upper layer of secretory and ciliated cells, even though the basal cells do not entirely underlie the more differentiated cells. To complete the picture, the epithelium also contains small numbers of neurosecretory cells, also called Kulchitsky's or K-cells (*4*). Although these cells may make a large qualitative contribution to the function of airway epithelium (for instance, K-cells may play a role in the regulation of mucin secretion), the remainder of this paper will focus upon the ciliated, basal, and mucosecretory cell types.

The ciliated cells provide the motive force for the so-called mucociliary escalator (*5*). Ciliated cells are a terminally differentiated cell type in the sense that such cells do not incorporate tritiated thymidine (*6*), and thus appear not to undergo cell division, which would be required for them to serve as a progenitor of other cell types. By contrast, both mucosecretory cells and basal cells are able to proliferate, again as judged by the criterion of tritiated thymidine incorporation (*6–8*), and thus either or both may be capable of further differentiation into other cell types. The fact that cells can be observed containing both mucous granules as well as cilia has been used to support the hypothesis that mucous cells differentiate further into ciliated cells (*1,2,6*).

The secretory cells of the upper airway can be histologically subdivided into several classes including Clara cells, small mucous granule cells, and goblet cells. The Clara cell is the most common nonciliated cell in the bronchiole. While this cell type does not occur as high as the trachea or bronchi in the human respiratory tree, marked species differences are seen, particularly in the rabbit, where Clara cells are a major cell type in the trachea (*3*). The cells very often protrude into the bronchiolar lumen and contain small, irregularly shaped granules that may or may not stain for carbohydrate depending upon species. The most common feature of Clara cells in all species is the abundance of smooth endoplasmic reticulum which may be indicative of either the secretory character of the cells, a role for the cells in detoxification of foreign substances by inducible cytochrome P-450 enzymes, or both (*9,10*). Recently, a 9–14 kD protein has been identified as the major secretory product of the Clara cells (*11*). While the function of this protein has yet to be established, its small size and absence of carbohydrate suggest that the secretory function of Clara cells is not simply a variant of that present in mucosecretory cells.

In goblet cells a large fraction of the cytoplasm in the apical region of the cells is given over to large granules that sometimes cause the apical plasma membrane to protrude into the lumen of the airway (*1,4*). A variety of staining techniques can demonstrate that such granules contain carbohydrate, which would be consistent with the presence of the highly glycosylated mucous glycoproteins or mucins. The glycoproteins are the major secretory product of tracheal epithelium and have the important functional property of conferring viscosity upon their aqueous solutions. This viscosity in turn contributes to the gel-like quality of the mucous layer that coats the epithelium and serves to entrap inhaled microbes and particulates. The gel with any such foreign material is swept up and out of the airway by the action of cilia.

A second cell type that also contains glycoconjugates is the small mucous granule cell, which is morphologically and histologically similar to mucous goblet cells except that its secretory granules are quite small relative to goblet cell granules. Like the goblet cell granules, the small mucous granules stain for carbohydrate, are membrane bound, and are apically positioned. Such cells have been proposed to be either a partially differentiated form of goblet cells or mature goblet cells early in the secretory cycle (*1*). In either case, goblet and small mucous granule cells seem to be closely related, both morphologically and functionally.

Pathways of Airway Cell Differentiation

In addition to the pathway of differentiation leading to the morphologically normal airway just described, an alternate pathway exists in pathological situations where the normal columnar mucociliary epithelium is replaced by squamous or epidermoid metaplasia. Such metaplasia can be induced by vitamin A deprivation (*8,12–15*) or toxic or mechanical injury (*6,7,16*). The product of squamous differentiation is a stratified epithelium with the top most layers of cells becoming flattened and ultimately forming cross-linked envelopes much in analogy to terminal epidermal differentiation in the skin (*17*).

Given that two distinct pathways of differentiation exist for airway epithelium, it is of great interest to know what factors control the expression of both mucociliary and squamous differentiation. Further, within the context of mucociliary differentiation, it is not clear what regulates the expression of the ciliated phenotype versus that of the goblet cell or small mucous granule cell. Finally, the morphogenetic relationships among the various major cell types are not clear. For instance, does the basal cell serve as the progenitor cell of the other three cell types (Fig. 1, Scheme Ia)? Or, does the basal cell and small mucous granule cell exist independently from each other; the basal cell giving rise only to basal cells and the small mucous granule cell functioning as the stem cell for the goblet and ciliated cell (Fig. 1, Scheme II)?

To address these types of questions, previous workers have used morphometric analysis of fixed tissue after manipulations of tracheas designed to influence differentiation (*6–8,12,16*). A second approach would be to use an *in vitro* culture system coupled with the biochemical measurement of a differentiation-related molecule. Ideally, such a system would be composed of an undifferentiated cell population that could be manipulated experimentally to give rise to differentiated cells, as measured by a specific differentiated function of the cells anal-

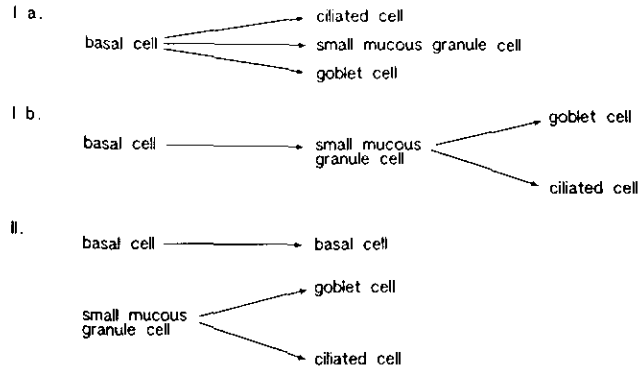

FIGURE 1. Possible interrelationships between different cell types in the tracheal epithelium.

ogous to what takes place *in vitro*. Early attempts (*18–23*) to generate cell lines derived from airway cells in culture were in general unsuccessful in that no specific differentiated functions were retained by the cell lines. Reports of cells in culture able to synthesize and secrete mucous glycoproteins (*20,21,24–27*) are open to the criticisms that the purported glycoproteins either have molecular weights inconsistent with that of mucin or were incompletely characterized. Wu and co-workers (*18,27,28*), applying techniques pioneered by Sato (*29,30*) in other systems, developed effective means to culture primary airway epithelial cells such that a proliferative population of cells could be grown to confluency. Cells were obtained from a protease digest of tracheal epithelium of rats, hamsters, or rabbits (*27*).

The cell population contained in the protease digest of rabbit trachea (RbTE) is composed of approximately 30% basal cells, 40% ciliated cells, 15% Clara cells, and 15% mucous cells (*31*), which compares favorably with abundances described morphometrically in rabbit trachea by Plopper and co-workers (*3*). Plopper et al. report values of 28% basal, 43% ciliated, 18% Clara, 1% mucous, and 9% unidentified. The cells placed in culture rapidly lose such differentiated characteristics as mucous granules and cilia (*28*) under culture conditions that have been optimized to favor attachment, proliferation, and the subculturing of the cells. The optimal medium includes insulin, transferrin, epidermal growth factor, and bovine hypothalamic extract. The RbTE cells can be grown on a variety of substrata, including untreated plastic, plastic treated with FAV (a mixture of fibronectin, albumin and Vitrogen, a brand of collagen I), or collagen gels, prepared from neutralized Vitrogen that has been permitted to form a thick (3–5mm) gel. On FAV-coated dishes, the cell population from the rabbit trachea will attach with an efficiency of about 15% to give rise to a proliferative cell culture with a population doubling time of about 19 hr. The cells are also quite migratory and apparently move apart after cell division occurs such that classical colonies are not formed. A typical dish of cells during the late exponential phase of growth just before stationary phase is achieved will be composed of many individual, rounded cells.

The nature of the proliferative cells obtained in culture is open to interpretation. As mentioned previously, the cells in early culture contain no morphologically recognizable differentiated features. Further, they display a high nuclear-to-cytoplasmic ratio reminiscent of basal cells. These data, combined with the fact that basal cells seem to be the most proliferative cells of the airway *in vivo*, might lead to the conclusion that the cells which proliferate *in vitro* are basal cells. An alternative interpretation might be that the initial mixed cell population has dedifferentiated in culture to form a mixed proliferative population which might in some way be induced to redifferentiate.

Recently, a 95% pure basal cell population has been obtained from mixed tracheal cell population by centrifugal elutriation (*31*). These cells have the same behavior in cell culture as the proliferative population derived from growing mixed cell populations. Further, both a mixed cell population and a 95% pure basal cell preparation have been used in experiments to repopulate a denuded tracheal implant. In this type of experiment (*32*), an excised trachea is stripped of its epithelium by freeze-thawing. Then the trachea is filled with a cell suspension, both ends are ligated, and the trachea is implanted SC in a host animal. After several days or weeks, the implant is removed and the regenerated epithelium of the trachea is examined morphologically. The 95% pure basal cells, the mixed cell population obtained from the trachea, and the proliferative cells that grow out from the mixed population *in vitro* can each give rise to a morphologically normal epithelium containing basal, mucous, and ciliated cells (*33*). This indicates again that the cells in culture have characteristics similar to basal cells and further, that both basal cells and the cells *in vitro* are multipotent, that is, able to give rise to other differentiated cell types. Rigorously, of course, it is not possible to completely exclude the option that a tiny fraction of the cells in the 95% pure basal cells give rise to the other differentiated type or that dedifferentiated cells *in vitro* could redifferentiate to repopulate tracheal implants in a normal manner; however, it seems reasonable to hypothesize that RbTE cells in the proliferative state in culture mimic basal cells and have the potential to differentiate into other cell types.

Given that RbTE cells *in vitro* have the potential to differentiate, what experimental manipulations could be used to realize that potential? It had been known for years, beginning with the classic observations of Wolbach and Howe (*34*), that vitamin A status has a profound effect upon the development and maintenance of normal epithelial differentiation. In airway epithelium, vitamin A deprivation leads to the expression of squamous or epidermoid metaplasia. An analogous change can be observed *in vitro*. Upon reaching confluency, RbTE cells cultured in the absence of vitamin A analogues will spontaneously differentiate to give squamous cells. These cells lose their proliferative capacity and express several markers of squamous differentiation including characteristic changes in keratin protein patterns, induction of a specific type I (epidermal) transglutaminase, increased activity of cholesterol sulfotransferase, and concommitant accumu-

lation of cholesterol sulfate, and ultimately formation of cross-linked envelopes by almost all the cells (*35–38*). All of these changes can be coordinately prevented by the inclusion of retinoic acid in the cell culture medium, indicating that RbTE cells are responsive to retinoids and that retinoids serve to prevent the entire program of squamous differentiation. Inhibition of squamous differentiation is not accomplished simply by maintaining the cells in a proliferative state, since both retinoic acid-treated cells and untreated cells lose proliferative capacity upon reaching confluency.

In contrast to the inhibition of squamous differentiation by retinoids, the process of squamous differentiation can be enhanced at early times in culture by a variety of experimental manipulations including withdrawal of EGF or insulin supplementation (*39*), or inclusion of transforming growth factor β (*40*) or phorbol esters (A.M. Jetten, unpublished results) in the culture medium. Most of these treatments seem to lead to a cessation of proliferation, followed by an induction of the squamous phenotype. Thus, the RbTE cell system has been a useful model to investigate squamous differentiation of the airway.

Although RbTE cells grown on FAV-coated dishes readily express squamous differentiation in the absence of retinoids, simple inclusion of retinoids in the culture medium does not lead to normal mucociliary differentiation. Therefore, other factors must be necessary for the expression of this pathway of differentiation. Wu and collaborators (*18*) had noted that hamster tracheal epithelial (HTE) cells grew poorly on uncoated plastic as a substratum. In contrast, increased colony-forming efficiency of the cells on collagen-coated plastic dishes was proportional to the collagen density. As the ultimate in collagen density, collagen gels were used as a substratum that led to attachment frequencies of 20 to 50% and rapid cellular proliferation with population doubling times on the order of 20 hr. Morphological analysis of confluent cultures of HTE cells by scanning and transmission electron microscopy demonstrated the presence of both ciliated cells and cells containing cytoplasmic granules near the cell apices. The granules were stained positively by methods designed to detect glycoproteins, suggesting that the granules contained mucins.

The ciliated cells observed in HTE cell cultures on collagen gel did not seem to be simply retained from the original cell population, but rather the original ciliated cells from the trachea declined in number during the proliferative phase and cilia subsequently reappeared after confluency was reached (*18*). This is consistent with the proposal that the proliferating HTE cells are undifferentiated and at confluency the cells differentiate to give rise to ciliated cells. For the expression of the ciliated phenotype the presence of conditioned medium from 3T3 fibroblasts was required. Conditioned medium is harvested from cultures of rapidly proliferating 3T3 fibroblasts and presumably contains factors secreted by these cells. Its requirement suggests that *in vivo*, the mesenchymal layer underlying the basement membrane of the airway epithelium may make a contribution to the growth and differentiation of the epithelium.

Use of Mucin as a Marker of Mucosecretory Differentiation

With morphological evidence in hand that HTE cells can express differentiated functions *in vitro*, a next logical step was the application of a biochemical criterion for mucociliary differentiation: specifically, did HTE cells synthesize and secrete mucous glycoproteins?

Mucins from several tissues and species exhibit several characteristics in common that can be used as criteria for the identification of mucins (*41*). These characteristics include high molecular weight, oligosaccharides linked to threonine or serine residues via *N*-acetylgalactosamine, and a sugar composition including *N*-acetylgalactosamine, *N*-acetylglucosamine, galactose, and possibly fucose and sialic acid, but excluding mannose. The high molecular weight of these molecules is due to a high degree of glycosylation (carbohydrate accounting for 50 to 90% of the molecular weight) of a large number of threonine and serine residues (which can account for 25 to 35% of the amino acid composition). This high degree of glycosylation is believed to give rise to a large sphere of hydration which in turn leads to an extended conformation generating the characteristic viscosity of aqueous solutions of mucins. In particular, the presence of sialic acid on mucin oligosaccharides has been shown to contribute greatly to the viscosity of mucin solutions (*42*).

In any cell culture system, only limited amounts of metabolic products will be obtained due to the relatively small numbers of cells present. Fortunately, the use of radioactive precursors to mucins can lead to significant amounts of radioactive incorporation and much useful information can be obtained. When HTE cells are incubated with [^{3}H]-glucosamine, radioactivity can be recovered from the medium that is excluded from a gel filtration column containing Sepharose CL-4B, consistent with a molecular weight greater than 10^6 (*43*).

The same chromatographic behavior is noted under reduced and denaturing conditions. The radiolabeled material is resistant to the action of several glycosaminoglycan-degrading enzymes, indicating that the cells do not produce hyaluronic acid or high molecular weight proteoglycan aggregates. Strong acid hydrolysates of this high molecular weight material contain [^{3}H]-*N*-acetylglucosamine and [^{3}H]-*N*-acetylgalactosamine. The conversion of [^{3}H]-glucosamine to sialic acid can readily be demonstrated, as the high molecular weight material is susceptible to the action of neuraminidase. The oligosaccharides containing the radioactive monosaccharides can be released from the polypeptide by reductive beta-elimination with the conversion of about one-half of the *N*-acetylgalactosamine to *N*-acetylgalactosaminitol, indicating that the oligosaccharide was originally linked via this residue.

Radioactivity can also be incorporated into HTE cell high molecular weight material from [^{3}H]-galactose, which is present as galactose in the oligosaccharides and from [^{3}H]-mannose, but all of this incorporation into oligosaccharides is present as [^{3}H]-fucose, derived from intracellular metabolism (*44*), while none is recovered as [^{3}H]-

mannose. Thus, the material secreted by HTE cells does fulfill the requirements established for characterization of mucin, including high molecular weight, monosaccharide composition, and linkage via *N*-acetylgalactosamine. A similar system of epithelial cell culture has been developed by Mendicino and co-workers (*45*) using a cell population derived from porcine trachea. These cells proliferate to confluency on dishes coated with 1% collagen, contain secretory granules, and secrete high molecular material with chemical properties of mucin. The mucin synthesized *in vitro* also cross-reacted with antibodies raised against mucin obtained from tracheal washes.

In principle, the HTE cell system might be used to investigate the differentiation of airway cells toward the expression of mucosecretory function. The cells synthesize mucous glycoproteins in a differentiation-dependent manner because proliferative HTE cells synthesize little mucin, whereas confluent HTE cells incorporate 30-fold more [^{3}H]-glucosamine into mucin (Fig. 2). In practice however, it is difficult to manipulate the HTE cell system such that differentiation is modulated. As already mentioned, the cells do not proliferate readily in the absence of collagen gel as a substratum; therefore, the effect of substratum upon differentiation cannot be evaluated. Likewise, any effect of retinoids is difficult to demonstrate due to the requirement for 3T3 cell-conditioned medium, which itself contains retinol derived from fetal bovine serum. Since RbTE cells will grow in serum-free medium, can be cultured upon several different substrata, and will respond to retinoids *in vitro*, it was decided to evaluate the production of mucins by RbTE cells.

As stated earlier, RbTE cells cultured on FAV either in the presence or absence of retinoids in the medium grow as a monolayer and do not undergo mucociliary differentiation (*35*). RbTE cells cultured on a type I collagen gel

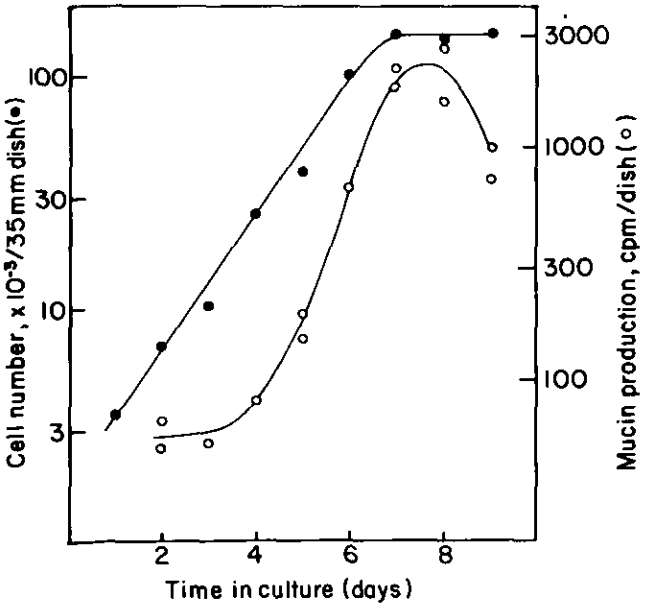

FIGURE 2. Mucin production by hamster tracheal epithelial cells in culture at different stages of the growth curve (*43*).

matrix either in the presence of absence of retinoic acid stratify. In the absence of retinoic acid the upper layers undergo a pathway of squamous cell differentiation as indicated by the appearance of squamous, cornified cells (*46*). Under this condition cells are rich in desmosomes and tonofilament bundles and express several biochemical markers associated with this phenotype. In the presence of retinoic acid, the upper layers consist of columnar, polarized cells that exhibit apical tight junctions. The cytoplasm of these cells is rich in rough and smooth endoplasmic reticulum and contains a well-developed Golgi apparatus and secretory granules that stain positively with Patch reaction and several lectins, indicating that they contain glycoconjugates. Morphologically, the cells resemble small mucous granule cells.

When RbTE cells were grown on either FAV-coated dishes or collagen gels, with or without retinoids, the cells incorporated [^{3}H]-glucosamine into material secreted into the medium that was excluded from gel filtration columns containing Sepharose CL-4B (*46–48*). In contrast to HTE cells, however, which contained no hyaluronidase-sensitive material, the high molecular weight material from RbTE cell medium exhibited variable amounts of hyaluronidase-degradable material. Cells grown on FAV-coated dishes secreted no hyaluronidase-resistant high molecular weight material either in the presence or absence of retinoids, indicating that on FAV, hyaluronic acid is the only high molecular weight glycoconjugate synthesized by the cells. Hyaluronic acid was also synthesized by cells grown on collagen gels, but a large fraction (35–45%) of the [^{3}H]-glucosamine-labeled high molecular weight material was resistant to the action of hyaluronidase and was separated from hyaluronic acid by ionic exchange chromatography. This ionic exchange-purified material was characterized as mucin by the same criteria used for the characterization of HTE cell mucin, namely, high molecular weight, carbohydrate composition (*N*-acetylglucosamine, *N*-acetylgalactosamine, galactose and sialic acid, but no mannose, or incidentally, fucose), and *N*-acetylgalactosamine as the linkage sugar. Additionally, the ion exchange chromatographic behavior of the material was sufficient to rule out glycosaminoglycans as potential contaminants.

The presence of retinoic acid in the medium greatly stimulates the production of mucin (Fig. 3) in agreement with the morphological observations. These findings are consistent with the interpretation that retinoids enhance mucosecretory differentiation in this cell system, whereas cells grown in the absence of retinoids follow a pathway of squamous cell differentiation. A schematic view of an interpretation is presented in Figure 4. Whether retinoids effect mucin production at the transcriptional or posttranscriptional level has to be established. Chytil and Ong (*49,50*) proposed that the action of retinoids is mediated via specific receptors that interact with specific sites at the chromatin, thereby altering gene transcription. This hypothesis is supported by recent findings identifying a nuclear receptor for retinoic acid (designated RAR) that appears to function like a steroid receptor (*51,52*). It is possible that the stimulation in mucin synthesis by retinoids is related to increased rate of transcription of the

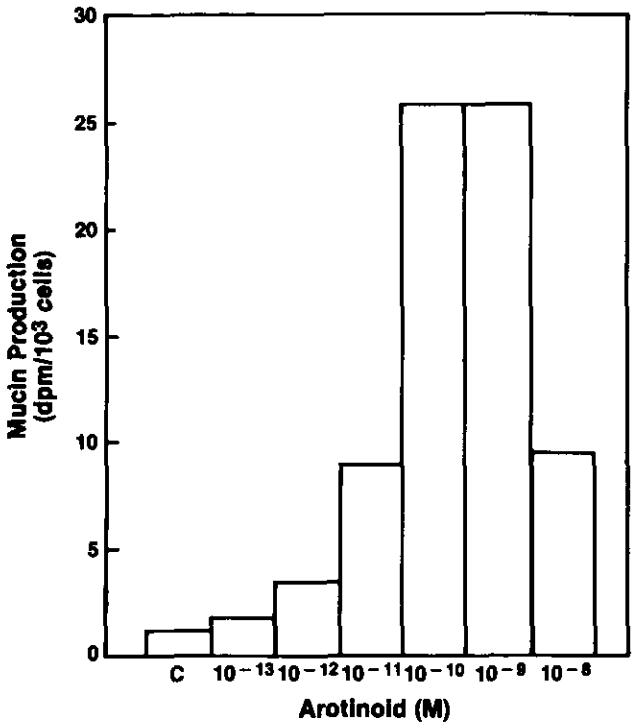

FIGURE 3. Production of mucinlike material by RbTE cells as a function of arotinoid concentration (*48*).

mucin gene mediated by the specific binding of the retinoic acid-RAR complex to a specific enhancer region of the mucin gene (Fig. 5).

Alternatively, retinoic acid could modulate mucin production in an indirect manner. For example, retinoic acid could induce the transcription of a gene coding for a transcriptional factor that regulates the transcription of the mucin gene. Isolation of the mucin gene will be necessary to distinguish between these possible modes of action.

The RbTE cell system has been used to identify some requirements of mucosecretory differentiation *in vitro* (*48*). Table 1 contains a summary of the effects of several parameters upon the production of mucin by RbTE cells in culture. Growth of the cells upon collagen gels seems to be an absolute requirement for mucin production. As mentioned, retinoic acid has a stimulatory effect. Several other substances that have been shown to stimulate differentiation in other systems (*53–55*), including butyrate, 5-azacytidine, and hexamethylenebisacetamide, were without effect in enhancing mucin production by RbTE cells, whereas 8-bromo-cyclic AMP more than doubled retinoid-induced mucin production. Interestingly, 8-bromo-cyclic AMP alone had no effect in enhancing mucin production. This action of cyclic AMP to stimulate retinoid-dependent differentiation-related parameters has now been observed in at least three other differentiation systems (*56–58*). The mechanism by which cyclic AMP increases differentiation in these systems may be by means of its classical action in activating protein kinase-catalyzed phosphorylation reactions or by way of a novel mechanism involving cyclic AMP-binding protein complexes that activate gene expression (*59*), perhaps in concert with the retinoic acid receptor mentioned above.

3T3 cell-conditioned medium, which had been shown to be required for the expression of ciliated cells by HTE cells (*18*), also had a stimulatory effect on mucin production by RbTE cells. This effect was enhanced by the inclusion of retinoids and 8-bromo-cyclic AMP in the culture medium. In summary, several medium components can be shown to regulate mucosecretory function in RbTE cells, but each requires that the cells be grown on collagen gel as a substratum before any mucin production can be measured.

The reasons substratum has such a profound effect upon mucin production are unknown. In mammary epithelial cell cultures (*60*), growth upon floating collagen gels is optimal for casein synthesis and secretion. Relative to cells grown on plastic or attached collagen gels, those grown on floating gels contain more casein mRNA, respond more readily to prolactin, which induces casein mRNA synthesis, and exercise less intracellular degradation of casein proteins once synthesized. Thus, mammary epithelial cells respond to collagen substrata at several levels to yield enhancement of a tissue-specific function. In the RbTE cell system, regulation of mucin production by substratum could likewise occur at the transcriptional, translational or posttranslational level.

One interesting difference between RbTE cells grown on coated plastic versus collagen gels is that the collagen

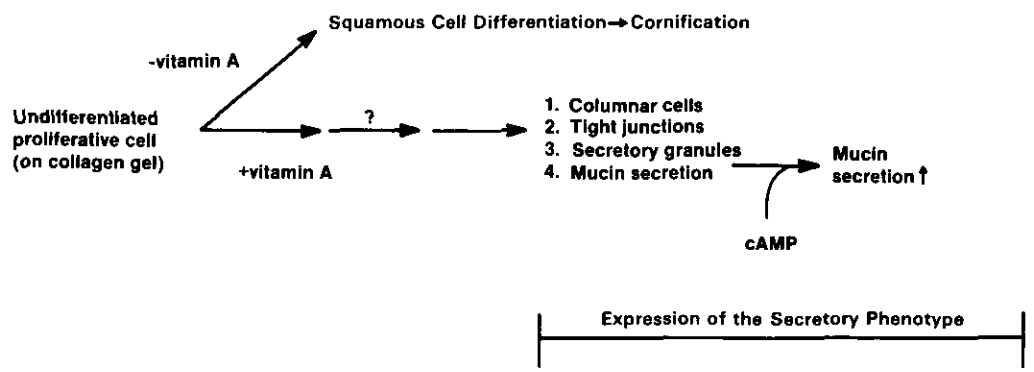

FIGURE 4. Schematic view of the differentiation process of RbTE cells *in vitro*. Cells grown on a collagen gel matrix and in the absence of retinoids undergo a pathway of squamous cell differentiation, whereas in the presence of retinoids, cells differentiate along a mucosecretory pathway.

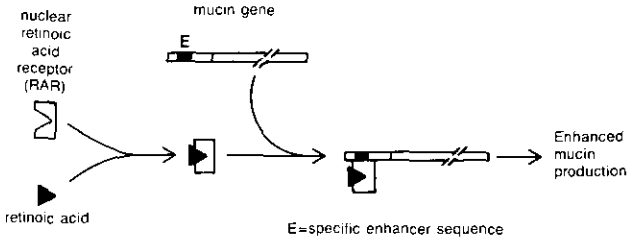

FIGURE 5. Schematic view of a possible mechanism by which retinoids regulate mucin synthesis.

gels permitted a stratified culture containing several layers of cells, while cells grown on coated dishes only form a monolayer (*46*). Obviously, cells in monolayer would have their basolateral surfaces in contact only with the coated surface that contains fibronectin, albumin, and collagen I. The same collagen I is used to form the collagen gel so that simple contact with that substance cannot account for differences in mucin production. Indeed, cells containing secretory granules are well separated from the collagen gel by a layer (3–6 cells thick) composed of cells with morphology quite reminiscent of the basal cells of the airway *in vivo*. It would seem the substratum for the cells producing mucin is not the collagen gel but rather the basal cells that are present in culture. Contacts between these layers, or perhaps factors secreted by the lower layers, may be necessary for the expression of differentiated functions of the upper layers.

In any case, collagen gels provide the matrix necessary for stratification into a multilayered culture. This *in vitro* organization is somewhat different of course from that expressed *in vivo*, where only a pseudostratified epithelium is present with basically all cell types in contact with the basement membrane. Perhaps the common feature between the *in vivo* and *in vitro* organization is the contact between basal cells and more differentiated cells.

The mechanisms whereby cell-substratum or cell-cell interactions might modulate gene expression and ultimately differentiation are completely unknown. It might be expected that such contacts would be analogous to cell surface receptor-ligand interactions that generate intracellular second messengers which in turn would ultimately regulate gene expression.

Alternatively, contacts might result in shape changes and alterations in the organization of cytoskeletal components within the cells. Changes in the family of intermediate filament proteins, keratins, are a well-known feature of epithelial cell differentiation (*61*). Changes in cytoskeletal organization could transmit signals to the nucleus to effect transcriptional changes (*62*). Alternatively, the cytoskeleton might affect the expression of differentiated functions by altering intracellular trafficking. In the case of mucosecretory cells, mucin mRNA must reach the rough endoplasmic reticulum, the nascent mucin polypeptide must be glycosylated while passing through the Golgi complex, and the fully processed mucin must be packaged in secretory granules that ultimately fuse with the apical plasma membrane during the secretion process. Cytoskeletal elements could be involved at any or all stages of this process. One would postulate that growth upon collagen gel leads to an appropriate cytoskeletal organization permissive for the secretory process.

In summary, it is clear that differences in substratum have a profound effect upon the expression of mucosecretory function of RbTE cells in culture. In addition to opening a means to study the ways in which a host of other factors affect mucin production, much more work is needed to explain the ways in which substratum affects cellular function.

Table 1. Production of mucin by rabbit tracheal epithelial cells under various conditions.

Experiment	Conditions[a]		Mucin production
	Substratum	Additions	dpm/10^3 cells[b]
1	FAV	None	ND[c]
		RA	ND
	Collagen gel	None	1.5
		RA	13.3
2	Collagen gel	None	1.5
		AR	20.2
		AR + sodium butyrate	19.5
		AR + 5-azacytidine	16.7
		AR + HMBA	20.3
		AR + 8-Br-cAMP	55.5
		8-Br-cAMP	1.4
3	Collagen gel	None	1.9
		Conditioned medium	9.5
		AR + conditioned medium	66.6
		AR + conditioned medium 8-Br-cAMP	82.0

[a]Retinoic acid, 10 nM (RA) or the arotinoid, (*E*)-4-[2-5,6,7,8-tetrahydro-5,5,8,8-tetramethyl naphthalen-2-yl) propen-1-yl] benzoic acid, 0.1 nM (AR) and/or conditioned medium were present on days 3–9 after plating. All other additions were present on days 6 and 7.

[b]Mucin production was determined by incorporation of [^{3}H]-glucosamine (days 7–9) into high molecular weight material, nonadherent to DEAE-Sephacel at 0.15 M NaCl (*48*).

[c]ND, not detectable.

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