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REVIEW

Contribution of *in vitro* culture methods for respiratory epithelial cells to the study of the physiology of the respiratory tract

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Contribution of in vitro *culture methods for respiratory epithelial cells to the study of the physiology of the respiratory tract. M. Jorissen, B. Van der Schueren, H. Van den Berghe, J.J. Cassiman.*

ABSTRACT: Different techniques for culturing respiratory epithelial cells have been developed to overcome the limitations of studies on *in vivo* and on bioptic material. However, each culture technique has its limitations, specifically concerning the expression of differentiated properties. These methods and limitations are described and discussed. Special attention is given to recent developments, which may resolve some of the current problems.

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The respiratory tract epithelium lines a major part of the internal surface of the organism and therefore provides it with a dynamic interface with the external environment and, as such, plays a major role in the protection of the body against that environment. To accomplish this function optimally, the respiratory tract has developed a mucociliary transport system that moves liquid, mucus and entrapped particles towards the oropharynx for expectoration or swallowing. Mucociliary transport comprises essentially three processes: firstly ion and water transport, secondly mucin production and secretion and thirdly co-ordinated ciliary beating. Each of these three functions is essential and mucociliary transport deteriorates when one is altered. Impaired ciliary beating by structural alteration of the cilia in primary ciliary dyskinesia [1–3], for instance, reduces the mucociliary transport rate, and the impaired activation of apical membrane chloride channels in cystic fibrosis [4, 5] apparently makes the mucus sticky and more prone to infections.

It is therefore not surprising that for many years the basic properties of these three functions have been the subject of many investigations. Studies of respiratory tract epithelial cells *in situ* or on fresh samples are limited however for various reasons. Firstly, the availability of representative tissue samples, particularly from humans, is limited. Secondly, the variability is high and the reproducibility low because of the variability of the external conditions *in vivo* and of the interindividual variability. Thirdly, the respiratory tract is continuously exposed to numerous toxic and infectious agents that may alter or impede each of the three transport processes. Given these limitations, the parameters that can be evaluated on fresh tissues are the mucociliary transport velocity and an estimate of mean ciliary beat frequency for the ciliary activity; the transepithelial potential differences and their response to amiloride and an estimate of the volume of fluid secreted for the water and ion transport; and mucus samples can be analysed physicochemically and biochemically.

For all the above mentioned reasons *in vitro* culture systems have been developed to overcome the limitations and problems of the *in vivo* studies or of those performed on bioptic material.

Cells can be cultured under different conditions with variable degrees of differentiation and cell growth. The main problems are the representativity of the cultured cells for the *in vivo* situation and the limitations on culture duration and growth capacities. Traditionally culture systems are divided into organ cultures, explant cultures and dissociated cell cultures. The first two contain both epithelial and non-epithelial cells. The dissociated cell culture systems comprise the feeder layer cultures, the pure epithelial monolayer cultures and the suspension cultures.

In most of the culture systems cells do not express the natural differentiated properties of respiratory epithelium. The production and secretion of mucins is almost non-existent, particularly for human cells [6, 7]. The cilia may deteriorate and disappear completely [6, 8], making *in vitro* studies on ciliary structure and function impossible. However, the electrophysiological properties are rather well conserved in culture [9, 10]. To overcome the limited cell growth and culture duration, immortalized cell lines have been established.

In the present review the different culture techniques will be briefly described and special attention will be given to the expression of respiratory differentiated properties, since this differentiation is a major limiting factor in the experimental use. This will be done more extensively for the dissociated culture systems. Recent, promising new methods will be evaluated.

Organ cultures

Organ culture aims at the maintenance or development of cells with normal structural and functional cellular interrelations [11]. Although growth is not a major characteristic, cell divisions are present, even in ciliated cells [12]. Organ cultures have been used in a variety of research domains such as toxicology, microbiology, carcinogenesis and respiratory biochemistry. The advantages of this technique are that foetal tissues will differentiate [13, 14] and that epithelial differentiation is maintained in adult organ pieces [15, 16]. Ciliated cells can remain in culture for longer than 2 months [17]. The major disadvantages of this method are the high variability, the low reproducibility and the cellular complexity. Specifically, this cellular complexity makes it difficult to determine whether a particular effect on cell morphology and physiology by an experimental condition is the result of a direct action on epithelial cells or whether it is an indirect effect *via* neighbouring non-epithelial cells such as fibroblasts, endothelial cells, inflammatory cells and smooth muscle cells.

Explant cultures

In the explant method small pieces of tissue (explants) are cultured on a substration under conditions which permit proliferation of epithelial cells and spreading of the outgrowth from the explant on the supporting substratum [18]. For respiratory epithelial cells this outgrowth reaches maximally 2 cm [19, 20]. The differentiation of the cells in the outgrowth is poor and depends on the proximity of the explant [19]. The number of ciliated cells diminishes away from the explant and at the outgrowth edges only squamous cells are present regardless of the substratum which may be plastic, collagen [21, 22] or endothelial cell-derived extracellular material [23]. Cells from the outgrowths can be used for propagation in dissociated, pure epithelial cell cultures [19, 22, 24, 25]. The major disadvantages of this technique are the presence of non-epithelial cells in the explant and outgrowth on the one hand, loss of differentiation and limited growth on the other.

Feeder layer culture

Culture of dissociated respiratory epithelial cells on irradiated or mitomycin-treated Swiss 3T3 fibroblasts [21, 26], has no major advantage over dissociated cell cultures since neither differentiation nor growth are significantly better. Moreover, the time consuming nature of the method, the variable quality of the feeder fibroblasts and the increased risk of viral infection are disadvantages of this culture procedure [21].

Monolayer culture of dissociated cells

During the last 10 years, many monolayer culture systems for dissociated respiratory epithelial cells have been developed. The essential characteristic is the pure epithelial nature of the cultured cells. Growth and differentiation depend on many culture conditions, such as origin of the tissue, culture medium supplements and substratum. Other elements of minor importance are the dissociation procedure, the growth medium and the incubation temperature. Monolayer cultures have been widely used for many purposes. The normal and pathological electrophysiological characteristics and regulation of electrolyte transport have been investigated, as well as the composition and regulation of mucin production and secretion in specific systems. Also, cellular differentiation and its implications for metaplasia and carcinogenesis have been the subject of many studies.

Growth

The growth capacities of non-transformed respiratory epithelial cells are limited to ±25 population doublings with a doubling time of 1–3 days, a culture span of 1 month and a maximum of 5 passages, irrespective of the tissue or species origin of the cells. This has been demonstrated for tracheal cells from hamsters [27–31], humans [21, 22, 24], rabbits [32–34], dogs [9, 35], ferrets [36] and rats [37] and for human nasal cells [6]. Other animals investigated are guinea-pig and monkey [38], domestic fowl [39] and swine [40].

The culture medium has no major influence on cell growth. The most widely used medium is Ham‘s F12; others use MCDB 151, LHC-9, RPMI, M199, a 1/1 combination of Ham‘s F12 and DMEM and of M199 and DMEM and conditioned medium of 3T3 cells. While the reproducibility is high and the variability low, the limited growth capacity and culture duration are major inconveniences for long-term studies. Therefore, transformed human cell lines have been developed. These immortalized cells can be cultured for more than 1 year and retain at least some electrophysiological characteristics [41–43].

Epithelial nature

Although isolation and culture of specific cell types such as Clara cells [44] and basal and goblet cells [39, 40, 45] is possible, most cultures are started from nonselected surface respiratory epithelial cells. Recently, monolayer culture techniques for human tracheobronchial submucosal glands have been developed [46, 47].

The epithelial nature of the cultured cells can be demonstrated by their morphology (polarization, microvilli, tight junctions), positive reactions with epithelial specific antibodies (anti-keratins), as illustrated in figure 1, and by redifferentiation into a normal respiratory type epithelium on de-epithelialized trachea implanted in athymic mice [33, 34, 48].

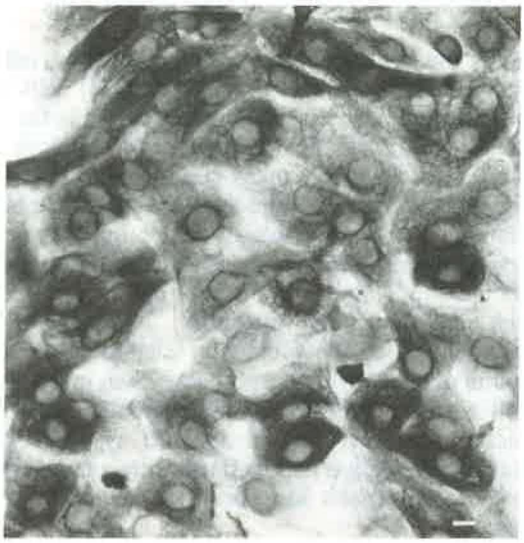

Fig. 1. – The epithelial nature of the cells dissociated from nasal polyps and cultured as a monolayer on a collagen gel is demonstrated by the positive reaction with keratin antibodies. Bar = 10 µm.

The expression of keratins in culture depends on the culture conditions and more specifically on the presence or absence of vitamin A [49, 50]. The pattern of keratins expressed is related to cell morphology and this may therefore be important in studies concerning differentiation, metaplasia and carcinogenesis.

Fibroblast contamination is not a major problem in respiratory epithelial cell cultures. It is reduced by low temperature [6, 27] enzymatic dissociation, for which pronase is usually chosen, and it can be reduced further by a preplating procedure in which the fibroblasts attach before the epithelial cells [8, 9, 34, 51], by serum-free culture media and by a culture temperature of 33°C instead of 37°C [20].

Fig. 2. – After 2 weeks the majority of the cells cultured as a monolayer are non-ciliated cells but some ciliated cells remain present (SEM). Bar = 10 µm.

Respiratory type differentiation in general

The two major differentiated cell types disappear almost immediately after plating at low density [37]: the goblet cells disappear first [31], followed by the ciliated cells [6, 31, 33, 37, 52]. However, after 2 wks some ciliated cells can still be present [8, 33, 53, 54] (fig. 2), (see below).

The expression of cellular differentiation in culture is largely dependent on the species, the culture media supplements and on the substratum. Briefly, the best results were obtained with hamster tracheal cells grown on a collagen gel in serum-free, hormone-supplemented medium [6, 28, 30, 38]. Cultured human respiratory epithelial cells neither express cilia nor mucin production [6, 7, 24] and retain only their electrophysiological properties [10, 55, 56].

The use of high (10–20%) serum concentrations [27, 30, 39, 40] has been abandoned because serum inhibits growth [21, 22] and induces squamous differentiation [6, 34, 57] possibly due to the presence of transforming growth factor β, [58, 59]. Serum is used for electrophysiological studies [9, 34, 51] and in lower concentrations (less than 2%) combined with multiple growth supplements for differentiation in hamster tracheal cells [28–30, 60, 61]. Recently, commercially available serum derivatives, such as NU-serum and Ultroser G, have been used with success for human nasal epithelial cells [8]. Most serum-free media are supplemented with insulin, epidermal growth factor, endothelial cell-derived growth factor and transferrin. Also choleratoxin, hydrocortisone, ethanolamine and phosphoethanolamine are added to enhance cell growth. Retinoic acid, in contrast, does not promote cell growth, but is necessary for the production of mucin-like glycoproteins and for the reappearance of cilia in hamster tracheal cells [7, 31, 32, 58, 62, 63].

Plastic has been used with success for culturing rabbit [34] and canine [53] tracheal cells and human airway cells [6, 7, 24], but it is inferior to collagen for mucoid differentiation in rabbit [60, 63], hamster [31] and human cells [6, 7] and also for the ciliary differentiation of hamster tracheal cells [30, 31]. Extracellular material [23] and laminin, albumin and fibronectin [6, 21, 22, 33] showed no advantage over plastic.

After reaching confluency, collagenolysis occurs [30] which can be reduced in serum-free media [31]; it can even be avoided by culturing at 32°C instead of 37°C [29, 61] or on collagen-coated millicell filters [28].

Permeable supports were initially used for electrophysiological studies [9, 10, 34, 55, 56]. Later it was shown that hamster tracheal cells become columnar in these substrata [28] and that the differentiation may be better for guinea-pig in the Whitcutt biphasic chamber [38]. This biphasic chamber contains a movable, transparent, permeable gelatine membrane on which the cells are grown and which can be placed at the air-liquid interface. In this culture system ciliated cells and mucin production are present after 8 days in culture [64].

Ciliated cells

One of the major disadvantages of monolayer cultures is the irreversible and total loss of ciliated cells (human, rabbit, rat, monkey, dog and ferret). The persistence of ciliated cells in monolayer cultures for a number of weeks has been observed in different species but only at high plating densities and mostly under restricted and well defined culture conditions [30, 31, 38, 53, 54, 64]. These remaining cilia, however, will often progressively degenerate and were formed *in vivo*, thus not during the culture period. Only in hamster tracheal epithelial cells [28, 30, 31, 38] and probably in guinea-pig tracheal cells grown in the biphasic Whitcutt chamber did cilia reappear [38]. For the reappearance of ciliated cells in hamster tracheal epithelial cells, a medium containing 2% serum, a collagen gel as substratum and 3T3 conditioned medium [30] were necessary. In serum-free, hormone-supplemented medium, ciliated cells were only seen in confluent cultures on a thick collagen gel in medium containing retinoic acid. Addition of serum then prevented the reappearance of ciliated cells [31]. Cilia also reappeared when cells were cultured on collagen-coated millicell filters in DMEM/M199 (1/1) with 5% serum and multiple growth factors [28] or in the conditions of the Whitcutt chamber [38].

The common and necessary conditions for reappearance of cilia are: a collagen substratum, confluent monolayers and a medium containing other supplements than serum alone. But this will lead to ciliogenesis only on hamster tracheal epithelial cells.

Mucins

A second major disadvantage of monolayer cultures, (and this specifically for human cells) is the absence or low expression of mucoid differentiation. Indeed, human nasal [6] and bronchial cells [7] cultured on plastic produce only hyaluronidase-sensitive proteoglycans, and this only in the presence of retinoic acid.

Hamster tracheal epithelial cells, cultured on plastic, produce and secrete a mucin-like glycoprotein according to one study [65], while another demonstrated only hyaluronic acid [31]. On collagen gels high molecular weight mucin-like glycoproteins were produced [62] and this depended on retinoic acid [24, 29, 31]. Further characterization revealed not only high molecular weight mucin-like glycoproteins, but also multiple proteoglycans, such as heparan sulphate proteoglycans and chondroitin sulphate proteoglycans, as well as different lipids, such as cholesterol, phospholipids and glycolipids [61]. These secreted products resembled very closely those found in mucus *in vivo*. Mucin release in confluent hamster tracheal surface epithelial cells is enhanced by fluid osmolality, pH changes and cationic proteases [66].

An influence of the collagen gel was also seen in cultures of rabbit tracheal epithelial cells: there was secretion of hyaluronic acid on plastic and mucin-like glycoproteins on collagen gel [60, 63]. Retinoids enhanced this mucin production, and this even more in a 3T3 conditioned medium with 8-bromo-cAMP [58, 63]. Recently, mucin production was demonstrated for guinea pig tracheal cells grown in the biphasic culture system [64].

In conclusion, expression of the mucoid differentiation is poor and restricted to hamster and rabbit tracheal cells grown on collagen gels and in the presence of retinoic acid.

Electrophysiology

The third characteristic of respiratory type differentiation is the presence of specific electrophysiological properties. Generally, the specific electrophysiological properties of respiratory epithelium are well conserved and expressed in culture taking into consideration the influence of the change in the cell shape on transepithelial properties [9, 10, 35, 53, 54]. Cultured cells from canine and human origin have frequently been used during the last 5 years to characterize the absorptive and secretory properties of respiratory epithelial cells [9, 10, 55, 67, 68]. Canine tracheal cells may retain ion transport properties resembling those of the original tissue for up to 2 months [51]. Specifically, the ion channels have been investigated with the patch clamp technique [4, 69, 70] and the defective regulation and activation of chloride channels in cystic fibrosis has certainly given more insight in normal and pathological electrophysiology [4, 5, 71].

Conclusions

Differentiation parameters such as cilia and mucin, are only poorly expressed in cultures of human nasal and/or tracheal epithelial cells. The only species in which cilia are repeatedly reported and production of mucin has been documented, is hamster. This poor expression of respiratory differentiated functions limits the possibilities for investigations of differentiation in general and of mucin production, secretion and regulation and of ciliary activity. However, recent developments in culture conditions (hormones, growth factors, permeable supports and culturing at the air-liquid interface) have shown that maintenance of cilia is possible for several weeks and that production and secretion of mucin-like components is measurable. Furthermore, the electrophysiological properties are well conserved in culture.

In addition to the studies on general differentiated properties, cilia and mucin production and regulation, monolayer cultures of dissociated surface respiratory epithelial cells have been used for investigations on carcinogenesis [72–74], virology [75, 76] and inflammatory processes [77, 78].

Suspension culture

Previously, it has been shown for many epithelia that functional and structural properties and differentiation are better preserved when the cells were grown as cellular aggregates in suspension than when cultured as monolayers [79–86]. However, suspension cultures of pure respiratory epithelial cells were only recently described [8, 87]. In suspension, these cells form stable aggregates, vesicles and spheroids, which remain for many months in culture, thereby exceeding the culture duration of nontransformed respiratory epithelial cells in monolayer culture by at least a factor 5. Manifest growth was not observed, as mitoses were not seen and as the diameter of the aggregates, vesicles and spheroids (and so the cell number) slowly but progressively decreased.

A major advantage of this method over the monolayer culture is the preservation of ciliated cells with normal ultrastructure and function for up to 9 months [8] (fig. 3).

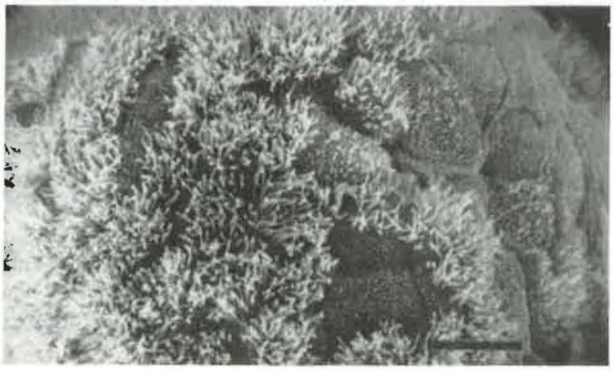

Fig. 3. – After 3 weeks suspension culture of dissociated nasal epithelial cells most of the cells are ciliated (SEM). Bar = 10 μm.

This suspension culture system has a second major advantage over the monolayer culture systems: cilia reappear after transfer of deciliated, flattened epithelial cells from the monolayer culture to the suspension culture system (fig. 4). This ciliogenesis is not only reproducible [8], but the cilia have a normal ultrastructure [8] and a co-ordinated, effective ciliary beating, as the aggregates, vesicles and spheroids continuously rotate in the culture medium [87]. In addition, the cytoplasmic precursor stages of the basal body formation and the ciliogenesis itself have been identified and indicate the same sequence of events [88] as has been observed during animal tracheal organogenesis and correlates with the few cytoplasmic precursor stages sporadically reported in human biopsies [89–92] and in trachea of human foetuses [93]. Finally with this technique the normal ciliogenesis, ciliary structure and function has been demonstrated in cystic fibrosis [94].

Starting from one single nasal biopsy one can culture a high amount of non-ciliated cells that will show ciliogenesis once brought in suspension. All these cilia are formed under well-defined conditions and without the influences of the unknown *in vivo* conditions.

Disadvantages of the suspension culture system include the impossibility to specifically influence the basolateral side of the cells and the continuous movements of the aggregates which interfere with observations on cilia of one cell. This suspension culture system may serve as a model for studies on the pathogenesis and classification of both primary and secondary ciliary dyskinesia. Also, the regulation of the ciliary differentiation can be investigated.

Before this system can be accepted as a complete model for differentiation of respiratory epithelium it still has to be shown whether there is production of mucins or of mucin-like glycoproteins.

Fig. 4. – When deciliated cells from monolayer cultures are released in suspension, aggregates, vesicles and spheroids are formed and after 1 week ciliated cells reappear. After 2 weeks in suspension ciliated and non-ciliated cells are present as well as cells in the process of ciliogenesis (SEM). Bar = 10 μm.

Conclusion

Various culture systems for respiratory epithelial cells are used to study airway epithelial functions, but each system has its unique advantages and limitations, which must be considered in the scope of specific experiments. Generally, in complex systems such as organ and explant cultures, a number of differentiated features of respiratory epithelia, such as cilia and mucins, are better preserved. Therefore these techniques are used for studying ciliary function and mucin production. However, the variability is high and intercellular interactions may contribute to the effects observed. In monolayer cultures of dissociated cells the electrophysiological properties and ion-transport systems are well preserved. Since only epithelial cells are present, the effects observed are caused by a pure epithelial response. Immortalized cell lines provide large quantities of cells but so far demonstration of a respiratory type differentiation is limited to the visualization of keratins and to electrophysiological properties. Suspension cultures of dissociated cells show that a complex system is not necessary for ciliary differentiation and this system can therefore be used to study cilia in a pure epithelial cell system.

In conclusion, a culture system in which all respiratory characteristics are expressed or maintained has not yet been developed and until then one has to rely on different, specific culture techniques to answer specific questions.

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Contribution des méthodes de culture in vitro *de cellules épithéliales respiratoires à l'étude de la physiologie du tractus respiratoire. M. Jorissen, B. Van de Schueren, H. Van den Berghe, J.J. Cassiman.*

RÉSUMÉ: Différentes techniques de culture de cellules épithéliales respiratoires ont été développées pour faire face aux limitations des études réalisées *in vivo* ou sur le matériel de biopsie. Toutefois, chaque technique de culture a ses limitations concernant spécifiquement l'expression des propriétés différenciées. Ces limitations sont décrites et discutées. Une attention particulière est donnée aux développements récents, qui pourraient résoudre quelques-uns des problèmes actuels.

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