Intermediate filament expression in normal and vitamin A depleted cultured hamster tracheal epithelium as detected by monoclonal antibodies

A study with emphasis on histological changes

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Summary. Using immunohistochemical techniques, the keratin expression patterns in basal and columnar cells (mucus-producing and ciliated cells) were investigated in tracheal organ cultures. Tracheas were from either hamsters fed a control diet or from hamsters fed a vitamin A-deficient diet; tracheas from the latter group were treated in vitro with all-trans retinol. In tracheas from hamsters fed a control diet, basal cells generally reacted with the RCK102 antibody and columnar cells with the RGE53 and the HCK19 antibodies, and both basal and columnar cells were recognized by the RCK105 antibody. The squamous cell cytokeratin 10 (detected by the RK5E60 antibody) was not expressed in cultured tracheas from hamsters fed a normal or a vitamin A-deficient diet. In the course of the in vitro period a number of keratins were ‘switched on’ or ‘switched off’ in both basal and columnar cells. In tracheas from vitamin A-deprived hamsters the RCK102 antibody clearly recognized basal cells and cigarette smoke condensate-induced proliferating basal cells, whereas the RGE53 antibody reacted with mucus-producing and ciliated cells. During organ culture foci of columnar epithelial cells expressed basal cell properties (detected with the RCK102 antibody) after all-trans retinol treatment and were found negative for the RGE53 antibody. Furthermore, it appeared that the RGE53-negative columnar cells contained periodic acid-Schiff-positive mucous granules. These findings indicate that basal cells may differentiate into columnar cells. Tracheal epithelium did not appear to co-express vimentin next to keratins during organ culture, which may be due to the intact three-dimensional organization present in these organ cultures.

Key words: Organ culture – Tracheal epithelium – Intermediate filaments – Vimentin – Keratin – Vitamin A

Introduction

The class of cytokeratins represents a morphologically homogeneous subgroup of intermediate-sized filament proteins specific to tissues of epithelial origin (Moll et al. 1982). The keratin expression patterns depend on epithelial cell type and stage of development or differentiation, and changes in these expression patterns may be related to the differences in molecular weights, isoelectric points and immunological properties of the individual keratin proteins (Franke et al. 1981; Moll et al. 1982; Ramakers et al. 1983).

Retinoids have an influence on proliferation and differentiation of epithelial cells and are essential for the maintenance of mucociliary activities in respiratory epithelium (Sporn and Roberts 1984). Previous studies showed that exposure to retinoids changes the cytokeratin expression patterns in different types of mammalian epithelial cells (Fuchs and Green 1981; Eckert and Green 1984; DeLuca et al. 1985; Huang et al. 1986; Wu and Wu 1986). Human keratinocytes cultured in medium supplemented with 10 to 20% serum containing vitamin A do not express the high molecular weight keratins (acidic 56.5 kDa and basic 65–67 kDa proteins). However, in the absence of
Fig. 1. Tracheal epithelium of vitamin A-deprived hamsters cultured in serum-free hormone-supplemented medium treated with DMSO, all-trans retinol or cigarette smoke condensate for several days. The tracheal cryostat sections were incubated with the monoclonal antibodies against intermediate filaments followed by an immunoperoxidase staining procedure (see Materials and methods). (A) Cultured tracheal epithelium treated with all-trans retinol \(10^{-7} \text{M}\) for 10 days. The left micrograph show RCK102-positive basal cells (arrowhead), and the right micrograph RGE535-positive mucus-producing and ciliated cells (arrow). Haematoxylin counter-staining, \(\times 225\). (B) Tracheal epithelium treated in vitro with all-trans retinol \(10^{-7} \text{M}\) for 6 days showing two daughter basal cells (arrow) which are RCK102-positive. One of the two cells (the most apically situated cell) shows a thin line of cytoplasm that migrates towards the lumen (arrowhead). Haematoxylin counter-staining, \(\times 225\). inset: enlargement of the two daughter basal cells, \(\times 500\). (C) Cryostat section of cultured normal tracheal epithelium treated with DMSO (1 ml/l) for 6 days showing RCK102-positive basal cells and some RCK102-positive cells in the mid-epithelial part containing lines of cytoplasm (arrowheads) that may migrate towards the tracheal lumen. Faint periodic acid-Schiff (PAS)-positive staining is present at the apical side of the epithelium (arrow). PAS counter-staining, \(\times 225\). (D) Tracheal epithelium obtained from vitamin A-deprived hamster treated in vitro for 6 days with retinol \(10^{-7} \text{M}\) showing several RCK102-positive mucus-producing and ciliated cells (arrowheads) and basal cells, \(\times 225\). inset: enlargement of RCK102-positive cells (arrow). Haematoxylin counter-staining, \(\times 500\). (E) Cultured tracheal epithelium obtained from hamsters fed a control diet, treated with DMSO (1 ml/l) for 6 days showing two divided basally situated cells (arrow) which are RCK102-positive. Some tracheal epithelial cells (arrowheads) which are positive show a cytoplasm that reaches to the tracheal lumen. PAS counter-staining, \(\times 225\). inset: enlargement of the two basally situated cells, \(\times 500\). (F) Vitamin A-depleted tracheal epithelium treated with cigarette smoke condensate (24 mg/l) for 10 days. Basal tracheal epithelial cell, two mucus-producing or ciliated cells (arrowheads) and a foetal of mucus-producing or ciliated cells (large arrow) are RCK102-positive. Faint periodic acid-Schiff (PAS)-positive staining is present at the apical side of the epithelium (small arrows). PAS counter-staining, \(\times 225\).

serum these keratins are expressed (Fuchs and Green 1981). Wu and Wu (1986) reported that retinoids inhibit the synthesis of the 48 kDa and 50 kDa keratin proteins and stimulate the synthesis of the 40 kDa and 52–54 kDa keratin proteins in cultured human bronchial epithelial cells. Furthermore, when hamsters are kept on a vitamin A-deficient diet changes in the keratin biosynthesis of tracheal epithelium are induced in organ culture (DeLuca et al. 1985; Huang et al. 1986). The squamous vitamin A-depleted tracheal epithelium expressed the 45, 46.5, 48, 50, 52, 55, 56 and 60 kDa keratin proteins, whereas normal mucociliated epithelium cultured in the presence of retinoic acid does not express these keratins. It has been suggested that retinoids influence keratin expression at the transcriptional level (Fuchs and Green 1981; Eckert and Green 1984).

We studied intermediate filament protein expression in cultured tracheal epithelial rings, with emphasis on the cellular proliferation and differentiation processes. Furthermore, we investigated the role of basal and proliferating basal cells in the genesis of differentiated tracheal epithelial cells, and the expression of vimentin.

Materials and methods

Animals and diets. Syrian Golden hamsters were obtained from the TNO Central Institute for the Breeding of Laboratory Ani-
Fig. 2. Serial cryostat sections of vitamin A-depleted hamster tracheas treated with all-trans retinol 10⁻⁷ M for 10 days. (A) Section showing a focus of mucus-producing or ciliated cells that are negative for RGE53 (arrowhead) surrounded by RGE53-positive cells. Basal epithelial cells are negative (arrow). Haematoxylin counter-staining, ×225. (B) Cryostat section showing that the RGE53-negative epithelial cells shown in A are RCK102-positive (arrowhead). Haematoxylin counter-staining, ×225

Fig. 3. Serial cryostat sections of vitamin A-depleted tracheal epithelium treated with all-trans retinol (10⁻⁷ M) for 10 culture days. (A) The micrograph shows a line of basal RCK102-positive cells and a focus of mucus-producing and ciliated RCK102-positive cells (arrow). Note the small mucus granule cells which are PAS-positive (arrowheads). PAS counter-staining, ×225. (B) Serial section showing that the mucus-producing and ciliated RCK102-positive cells shown in A are RGE53-negative (arrow) and are for the greater part small mucus granule cells which are PAS-positive (arrowheads). PAS counter-staining, ×225

Tracheal organ culture. Tracheal rings were cultured for up to 10 days in a serum-free CMRL-1066 medium (Flow Laboratories, Rickmansworth, UK) supplemented with L-glutamine 2 mM (Flow), hydrocortisone 0.1 mg/ml, bovine pancreas insulin 1.0 mg/ml (Sigma Chemicals, St. Louis, MO, USA) and gentamicin 50 mg/ml (Flow). Cultures were rocked eight to nine times per minute in a humidified incubator at 37°C in 5% CO₂, 45% N₂ and 5% O₂ (Rutten et al. 1988a). The vitamin A-depleted tracheas were treated with 1 ml/l dimethyl sulfoxide (DMSO) or with all-trans retinol (Fluka AG, Buchs, Switzerland) dissolved in DMSO at concentrations of 10⁻¹¹ and 10⁻⁷ M. Cigarette smoke condensate (CSC) was used at a final concentration of 24 mg/ml to induce basal cell proliferation in vitamin A-depleted tracheal epithelium (for preparation of CSC, see Rutten and Wilmer 1986). The control tracheas were treated with 1 ml/l DMSO.

Characterization of the monoclonal intermediate filaments antibodies. The following monoclonal antibodies against intermediate filament proteins were used: RGE53 (against human cytokeratin (HCK) 18); RCK560 (against HCK 10); RCK102 (against HCK 5 and HCK 8); RCK103 (which recognizes keratins and specifically stains basal cells in several organs; Ramakers et al. 1987); RCK105 (against HCK 7); clone 462 (against HCK 19; Bio-Makor, Rehovot, Israel); and vimentin (against swine vimentin, recognizes human and rodent vimentin; Dakopatts, Glostrup, Denmark). The classification of the keratins used has been published by Moll et al. (1982). More detailed information on the characteristics and specificities of the monoclonal intermediate filaments antibodies has been published previously (Ramakers et al. 1987).

Immunohistological staining. The tracheal rings were snap-frozen in isopentane at ca. −160°C, and stored in liquid nitrogen. Cryostat sections (7 μm) were mounted on glass slides which were pre-coated with poly-L-lysine, air-dried and fixed for 10 min in acetone.

The fixed cryostat sections were rinsed in phosphate-buffered saline (PBS, 0.01 M, pH 7.4) and incubated with the primary antibody (undiluted culture supernatant, except for HCK19 which was diluted 1:40) at 20°C for 1 h in a moist chamber, rinsed for 30 min in PBS, then incubated for 30 min with a peroxidase-conjugated rabbit anti-mouse immunoglobulin (Dakopatts, Glostrup, Denmark), diluted 1:200 in PBS, rinsed in PBS followed by Tris/HCl (0.05 M, pH 7.6), and finally incubated for 10 min with the chromogen diaminobenzidine as described by Polak and Noorden (1983). The sections were counterstained with Gill's Haematoxylin activity 3 (Polyscience, Warrington, FL, USA), or stained with periodic acid-Schiff (PAS) (Merek, Darmstadt, FRG).

Results

The epithelium of cultured tracheal rings showed a pseudostratified columnar epithelium expressing different cytokeratins depending on the degree of differentiation (Figs. 1–3 and Tables 1, 2).
Table 1. Expression of intermediate filaments in cultured tracheal epithelium from control hamsters

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B: basal cells  
M-C: mucus-producing and ciliated cells

* + + + proportion of peroxidase-positive cells greater than 90%  
+ + proportion of peroxidase-positive cells between 40 and 90%  
+ proportion of peroxidase-positive cells between 10 and 40%  
+/- proportion of peroxidase-positive cells between 1 and 10%  
0 proportion of peroxidase-positive cells smaller than 1%

Table 2. Expression of intermediate filaments in cultured vitamin A-depleted hamster tracheal epithelium treated with retinol $10^{-7}$ M

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B: basal cells  
M-C: mucus-producing and ciliated cells

* + + + proportion of peroxidase-positive cells greater than 90%  
+ + proportion of peroxidase-positive cells between 40 and 90%  
+ proportion of peroxidase-positive cells between 10 and 40%  
+/- proportion of peroxidase-positive cells between 1 and 10%  
0 proportion of peroxidase-positive cells smaller than 1%

RCK103. The keratin(s) detected by the RCK103 antibody are present in all epithelial cell layers, in both control tracheas treated with 1 ml/l DMSO and in vitamin A-depleted tracheas treated with $10^{-7}$ M all-trans retinol. This expression pattern is maintained during the whole organ culture period (Tables 1, 2).

RKSE60. In control tracheas, and in vitamin A-depleted tracheas treated in vitro with all-trans retinol ($10^{-7}$ M), the development of squamous metaplasia is inhibited. This histomorphological observation is supported by a RKSE60-negative reaction in the epithelium of all organ cultures indicating that no (single cell) keratinization occurs (Tables 1, 2).

HCK19. Immunostaining with the antibody against HCK19 in control tracheas shows that the basal epithelial cells are negative for this antibody, while the mucus-producing and ciliated cells are strongly positive (Table 1). In vitamin A-depleted
tracheal epithelium cultured for 2 days in the presence of retinol (10⁻⁷ M), a significant number of basal cells become positive with this antibody, whereas the mucus-producing and ciliated cells seem to stain somewhat less intensely than those of control epithelium. After the addition of retinol (10⁻⁷ M) to these vitamin A-depleted tracheas, HCK19 expression occurs only in the mucus-producing and ciliated cells after 10 days, which is comparable to the HCK19 expression found in control tracheas on culture day 2 (Tables 1, 2).

**RGES3 and RCK102.** The expression of keratins detected by the antibodies RGES3 and RCK102 in control tracheal epithelium remains almost unchanged during the whole culture period (Table 1). RGES3 stains the mucus-producing and ciliated cells and is virtually negative in the basal cells, but RCK102 reacts mainly with basal cells and leaves most of the mucus-producing and ciliated cells unstained (Fig. 1A). In vitamin A-depleted tracheas treated in vitro with a biologically active retinol concentration, however, a number of changes are observed with respect to the staining patterns of RGES3 and RCK102 (Table 2, Figs. 1–3). The addition of retinol (10⁻⁷ M) to vitamin A-depleted tracheal rings increases the number of RCK102-positive mucus-producing and ciliated cells between culture days 2 and 6. On day 10 the number of RCK102-positive mucus-producing and ciliated cells is decreased. On day 6 a significant increase in the number of RGES3-positive basal cells is seen, compared with days 2 or 10 (Table 2).

Figure 1A (left) shows a lining of RCK102-positive basal epithelial cells and RGES3-positive mucous-producing and ciliated cells (Fig. 1A, right) in a vitamin A-depleted trachea that had been treated in vitro with retinol (10⁻⁷ M) for 10 days. This staining pattern of RCK102 is comparable with the basal cell staining reaction found in control tracheal epithelium (Table 1). The number of RCK102-positive mucous-producing and ciliated cells is increased on day 6 in vitamin A-depleted tracheal epithelium treated with all-trans retinol (10⁻⁷ M; Fig. 1B, D), DMSO (1 ml/l; Fig. 1C) or cigarette smoke condensate (24 mg/l; Fig. 1F). Interestingly, two basal epithelial daughter cells are both RCK102-positive, and the most apically situated cell shows a thin line of RCK102-positive cytoplasm reaching towards the tracheal lumen (Fig. 1B, D, E). In serial cryostat sections, areas with RCK102-positive mucus-producing and ciliated cells are complementary to areas of RGES3-negative cells (compare Figs. 2A, B, 3A, B). Most of these cell groups are found to contain faint PAS-positive small mucous granules (Fig. 3A, B). Upon electron microscopic examination of acetone-fixed tracheal rings a few of these RCK102-positive non-basal cells appear to be ciliated cells (data not shown).

**RCK105.** In control tracheas virtually all epithelial cells are RCK105-positive at the beginning of the culture period (Fig. 4A). However, during organ
culture the number of RCK105-positive basal cells decreases (Fig. 4B), whereas the mucus-producing and ciliated cells remain RCK105-positive (Table 1). In contrast, vitamin A-depleted tracheal epithelium shows many RCK105-negative basal cells at the beginning of the culture period. The addition of retinol results in an increasing number of RCK105-positive basal cells during the organ culture period (Table 2; Fig. 4C). No changes are observed in the staining patterns of mucus-producing and ciliated cells with the RCK105 antibody during the 10-day culture period (Fig. 4A–C).

Epithelial cells of tracheal rings obtained from normal and vitamin A-deprived hamsters do not co-express the intermediate filament vimentin besides the various keratins during the 10-day culture period, whereas the mesenchymal cells (fibroblasts in the lamina propria and cartilage cells) are vimentin-positive (Tables 1, 2).

Discussion

There is wide agreement on the essential role of retinoids, especially retinol and retinoic acid, in the regulation of cellular proliferation and differentiation in tracheal epithelium (Sporn and Roberts 1984; McDowell et al. 1984a, b; Rutten et al. 1988a–c). The relationship between cytokeratin expression and vitamin A-deprivation in hamster tracheal epithelium has been reported recently (Huang et al. 1986; DeLuca et al. 1985; Rutten et al. 1988d). Huang et al. (1986) found that keratin biosynthesis increases in vitamin A-depleted squamous epithelium as detected by immunoblot analysis with polyclonal antibodies. DeLuca et al. (1985) reported similar changes in keratin expression as a result of vitamin A deficiency and benzo[a]pyrene exposure. Other workers have reported that retinoids influence keratin expression at the transcriptional level (Fuchs and Green 1981; Eckert and Green 1984). Recently, two studies reported the finding of a human retinoic receptor belonging to the family of nuclear receptors (Giguere et al. 1987; Petkovich et al. 1987). Moreover, a group of closely related retinoic acid receptor-related genes have been found to show also an affinity for retinol. Petkovich et al. (1987) suggested a conversion of retinol into retinoic acid before binding to the receptor. This important discovery shows a direct interaction of retinoids (retinol and retinoic acid) with DNA, and may indicate that retinoids influence keratin expression at the DNA level.

Concerning the monoclonal antibody RCK102 (which recognizes human keratins 5 and 8), there are major differences in staining patterns between human epithelium (Ramaekers et al. 1983) and cultured hamster tracheal epithelium as found in the present study. In human tracheal epithelium both keratins 5 and 8 are expressed (Moll et al. 1982). Therefore, in hamsters the RCK102-antibody is not necessarily specific for the hamster counterpart of the corresponding human keratin. It could be possible that antibody RCK102 recognizes only one of the human keratins in hamster tracheal epithelium.

The tracheal epithelium, both in vivo and in organ cultures, consists of a lining of pseudostratified epithelium. Although it is still unknown which pathways are important in the morphogenesis of differentiated tracheal epithelial cells, some authors indicate that both basal (Lane and Gordon 1979; Chopra 1982; 1983; Inayama et al. 1988) and small mucous granule cells (McDowell et al. 1984a, b; Sigler et al. 1988) play a role in this process. The results obtained in the present study indicate that, in addition to small mucous granule cells, dividing basal epithelial cells in the trachea give rise to columnar cells. Furthermore, in previous studies we have reported that ciliated cells in tracheal organ cultures can proliferate (Rutten et al. 1988c), and that CSC-exposure and vitamin A-depletion produce distinct basal cell proliferation (Rutten et al. 1988b). The fact that vitamin A-depleted mucus-producing and ciliated cells express basal cell properties after retinol and CSC treatment (as concluded from the RCK102-positive and the RGE53-negative reaction) suggests: (1) an increased rate of differentiation of basal cells into mucus-producing and ciliated cells, or (2) a switch-on of the RCK102-keratin and a switch-off of the RGE53-keratin in genuine columnar cells under the conditions used. Furthermore, it appeared that there are two types of PAS-positive small mucous granule cells: (1) cells that are recognized by RCK102-positive/RGE53-negative staining, and (2) cells that are RCK102-negative/RGE53-positive. It is suggested that the first group of small mucous granule cells are immature columnar cells still containing the basal cell keratin pattern, and that the second group are more mature, terminally differentiated, small mucous granule cells which have lost the basal cell keratin. In view of these findings, we assume a role for basal cells in the morphogenesis of differentiated columnar tracheal epithelial cells.

In recent years several studies have reported co-expression of keratin and vimentin in certain
normal tissues, in tumours, during embryonic development, and especially in cell cultures (Virtanen et al. 1981; Lane et al. 1983; Ramaekers et al. 1983; Emura et al. 1986; Gatter et al. 1986; Paranko and Virtanen 1986; Gröne et al. 1987). However, in the present study co-expression of vimentin and keratin was not observed in the tracheal epithelium of organ cultures, at least during the 10-day culture period of control epithelium, or in vitamin A-depleted epithelium treated with all-trans retinol. Moreover, neither CSC-treated nor vitamin A-depleted tracheal epithelium co-expresses vimentin and keratin as detected by immunohistological techniques (Rutten et al. 1988d). Respiratory epithelial cells in vitro may, however, co-express cytokeratin and vimentin (Emura et al. 1986), whereas vimentin is not detectable in non-cultured tracheal epithelium. A possible explanation for the absence of vimentin in the epithelium of tracheal organ cultures has been given by La Rocca and Rheinwald (1984), who suggested that cell-to-cell interactions in the three-dimensional organization of cells suppress the expression of vimentin in addition to keratin. Considering the mechanisms of action, experiments with cells in monolayers have pointed to a role for cellular prolifereation or desmosomal disruption in co-expression (Ben-Ze’ev A 1986; Emura 1986). Furthermore, our findings with tracheal organ cultures do not indicate that all-trans retinol influences the co-expression of vimentin in the epithelium. Therefore, we conclude that organized tissue structure along with cellular interactions and contacts may prevent the induction of vimentin expression in tracheal epithelium in organ culture.

In conclusion, the results obtained in the present study indicate that columnar tracheal epithelial cells may derive from basal cells. Furthermore, the RCK102 and the RGE53 antibodies seem to be useful tools for studying the role of basal cells in the restoration of mucociliary epithelium from vitamin A-depleted hamster tracheas. In tracheal organ cultures co-expression of cytokeratin and vimentin was not observed. This may be due to the intact three-dimensional organization present in these organ cultures.

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