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The morphological substrate of autonomic regulation of the bronchial epithelium

S. Philippou, H.J. Sommerfeld, M. Wiese, K. Morgenroth

Department of Pathology, Universitätstrasse 150, Ruhr-University of Bochum, D–44780 Bochum, Germany

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Abstract. Observations of explanted bronchial mucosa show that ciliary function is maintained for 7 days subsequent to explantation. This finding demonstrates that non-neural mechanisms exist which regulate ciliary function. Ultrastructural and immunohistochemical studies both for light and electron microscopy were performed on human bronchial biopsy material and lung resection specimens in order to recognize the morphological substrate of this regulatory mechanism. A complex system of cytokeratin filaments and microtubules radiate through the whole cytoplasm of ciliated cells with direct contact to the nucleus, cilia, microvilli, desmosomes and to the apical terminal adhesive complex. Between the basal bodies and the apical terminal adhesive complex microfilaments can be found. In the apical cytoplasm a dense filamentary network is seen in association with the adhesive complex. These morphological findings indicate that the cytoskeleton of the bronchial epithelium plays a key role in the co-ordination of ciliary function.

Key words: Ciliary regulation – Cytoskeleton – Bronchial epithelium – Electron microscopy

Introduction

Amongst the defence mechanisms of the lung the bronchial cleansing mechanisms are of special significance. For the effective function of this system a co-ordinated and adequate ciliary beat and the composition of the secretions on the surface of the bronchial mucosa are of key significance (Philippou et al. 1993b). The ciliary beat, the composition and amount of the secretions are precisely co-ordinated and in equilibrium with one another. In this complex interplay even slight changes in individual components may cause a loss of balance, impairing mucociliary clearance and promoting the effect of exogenic agents on the bronchial mucosa.

Correspondence to: S. Philippou

The exact mechanisms responsible for an effective, co-ordinated ciliary beat are not known. It is assumed, that ciliary function is regulated by the bronchial nerve supply and that the unmyelinated post-ganglionic nerve fibre endings of the vagus nerve play the main role (Morgenroth 1992). Our own studies of bronchial wall specimens in tissue culture medium obtained from human surgical and biopsy material (Philippou et al. 1993b, c) as well as bronchial wall probes from capsaicin-depleted animals (Philippou et al. 1993a) indicated that co-ordinated and adequate ciliary function is possible despite interruption of innervation. These findings indicate that the ciliary function is determined by a further regulatory mechanism, functioning independently of the nerve supply of the bronchial mucosa. An intra-epithelial or intracellular regulatory mechanism must thus be postulated. The sliding filament model (Satir 1974) is suitable for explaining the development of the ciliary beat, however, it is inadequate to explain the co-ordinated function of the cilia.

In ultrastructural studies of human bronchial mucosal specimens supported by immunohistochemical methods for light-, laser-scanning and electron microscopy. We wished to determine whether the morphological substrate of a mechanism responsible for the co-ordination of the ciliary beat is present in the bronchial epithelium.

Materials and methods

Fifty human bronchial wall specimens obtained by bronchoscopy from non-smoking patients were fixed in 2.5% glutaraldehyde and examined by transmission electron microscopy. Semi-thin sections of each specimen were examined prior to further study in order to assess their condition and suitability for our studies. Frozen sections of the lobe bronchi of 18 lung resection specimens were also prepared for immunofluorescence and laser-scanning-microscopic studies. Further bronchial wall probes were taken out of the 18 lung resection specimens, fixed in 4% formaldehyde and paraffin-embedded for immunomarking in histological sections for scanning electron microscopy.

Further probes were fixed in a combined 2.5% formaldehyde and 0.5% glutaraldehyde solution and embedded in London Resin

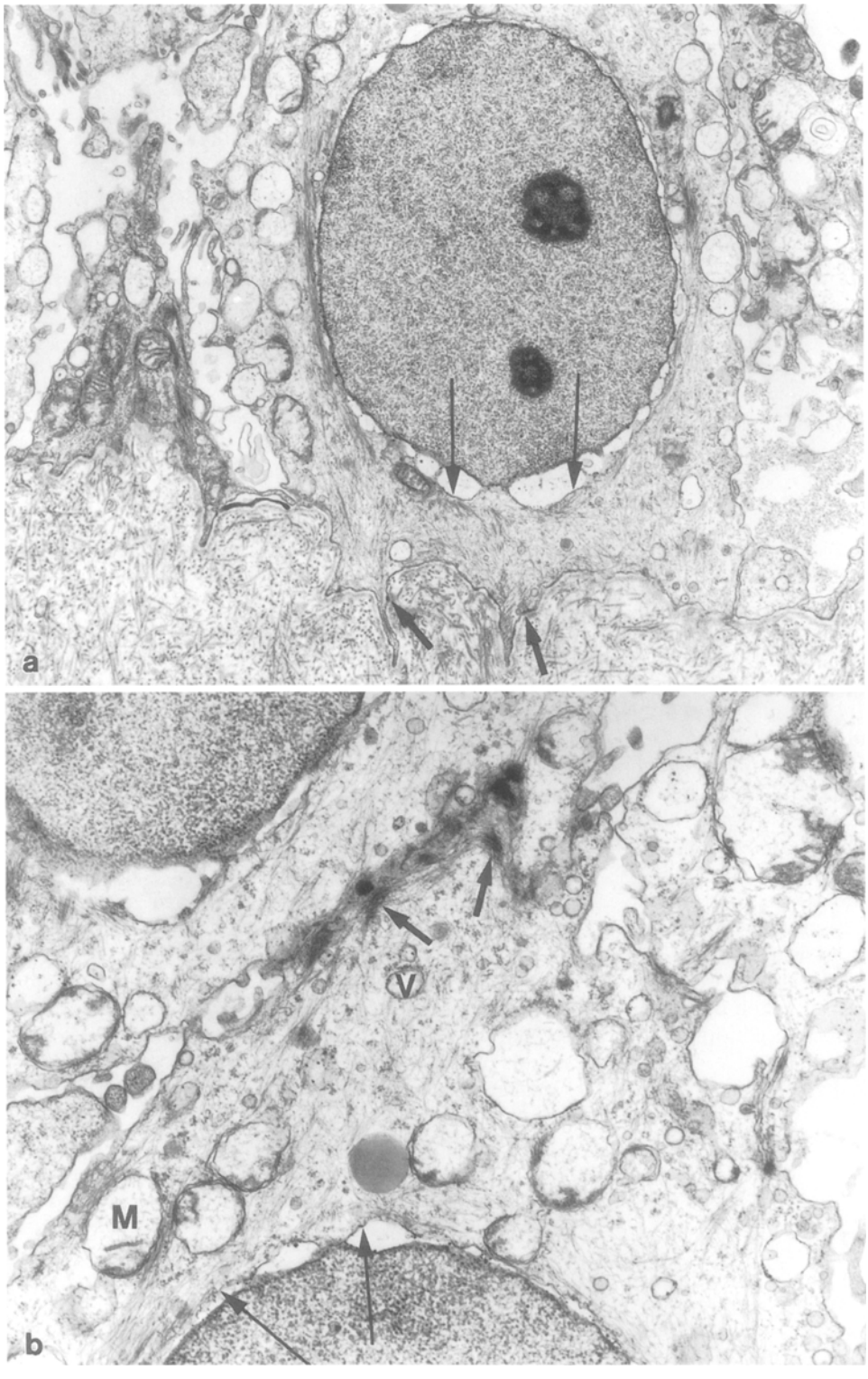

Fig. 1a. Basal cell of the bronchial epithelium. Branched, root-like cytoplasmic projection reaching deep into the basement membrane. The bundles of intermediate filaments developed in the caudal basal cell region radiate from the nuclear membrane *(narrow arrows)*, reach into the periphery and are in contact with hemi-desmosomes *(thick arrows)*. Transmission electron microscopy (TEM), 15000 X. **b** The bundles of intermediate filaments radiate from the outer nuclear membrane *(narrow arrows)*, reach into the periphery and are in contact with desmosomes *(thick arrows)*. The mitochondria *(M)* and the vesicles *(V)* are interspersed in the intermediate filamentary system. TEM, 39000 X

(LR)-White for immunoelectron microscopy. The 18 lung resection specimens all had peripheral carcinoma of the lung. In all the selected bronchial wall probes minimal inflammatory cellular infiltration was evident in the subepithelial zone.

For transmission electron microscopy (TEM) the specimens were embedded in Epon 812 and LR-White and prepared as usual for TEM. Immunoelectron microscopy was performed according to the "post-embedding" method (Polak and Varndell 1984) using IgG-conjugated gold 10 nm (Plano, Marburg-Cappel, FRG).

The immunofluorescence reactions on frozen sections for light- and laser-scanning microscopy were performed as usual by the fluorescein isothiocyanate-technique.

Immunomarking of histological sections for scanning electron microscopy were performed according to the method described by Philippou et al. 1992. Anti-pan-cytokeratin antibodies of the following companies were used for immunomarking:

1. BioGen Laboratories, Dublin, USA,
2. Boehringer Mannheim, Mannheim, FRG
3. Dianova, Hamburg, FRG.

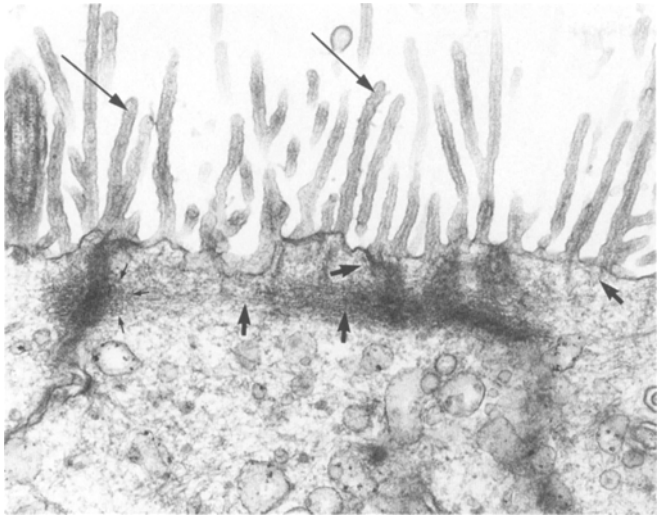

Fig. 2. Microfilaments and microtubules *(thick arrows)* run out of the apical filament- and microtubules system into the microvilli. The microtubules reach to the tip of the microvillus *(narrow, long arrows)*. In vicinity of the adhesive complex transversely sectioned microtubules *(narrow, short arrows)*. TEM, 59400 X

Negative controls for each series of sections which were immunomarked, were performed. The primary antibody was substitute with phosphate buffered saline (PBS), pH 7,4 (Sigma Diagnostics, St. Louis, USA).

The TEM EM 10 (Zeiss), the scanning electron microscope (SEM) DSM 950 (Zeiss) and the confocal Laser-Scanning-Microscope (Leica) were used.

Results

A multiplicity of individual findings allows us to reconstruct the cytoskeleton of the bronchial epithelial cells, especially the ciliated cells.

In vicinity of the basal cell layer of the epithelium the cells reach deep into the basement membrane by means of highly branched, root-like cytoplasmic projections, thus enlarging the contact surface between epithelium and basement membrane. Hemi-desmosomes are developed between the epithelial cells and the basement membrane. The bundles of intermediate filaments located in the caudal basal cell areas have direct contact with the hemidesmosomes.

The nucleus is surrounded by a variably dense reticular network consisting of filamentary proteins (Fig. 1a, b). These filaments measure 8 to 10 µm in diameter and some are bundled (tonofibrils) and are identified as intermediate filaments. The intermediate filaments are directly associated with the outer membrane surrounding the nucleus, which faces the cytoplasm (Fig. 1a, b).

The intermediate filaments radiate in variable thick bundles in a mainly reticular, net-like arrangement throughout the whole cytoplasm, into the periphery of the cell. The tonofibrils vary in thickness. The cell organelles lying in the cytoplasm are interspersed in the intermediate filamentary system (Fig. 1a, b), some having direct contact to the individual filaments. The intensity of the contacts varies greatly.

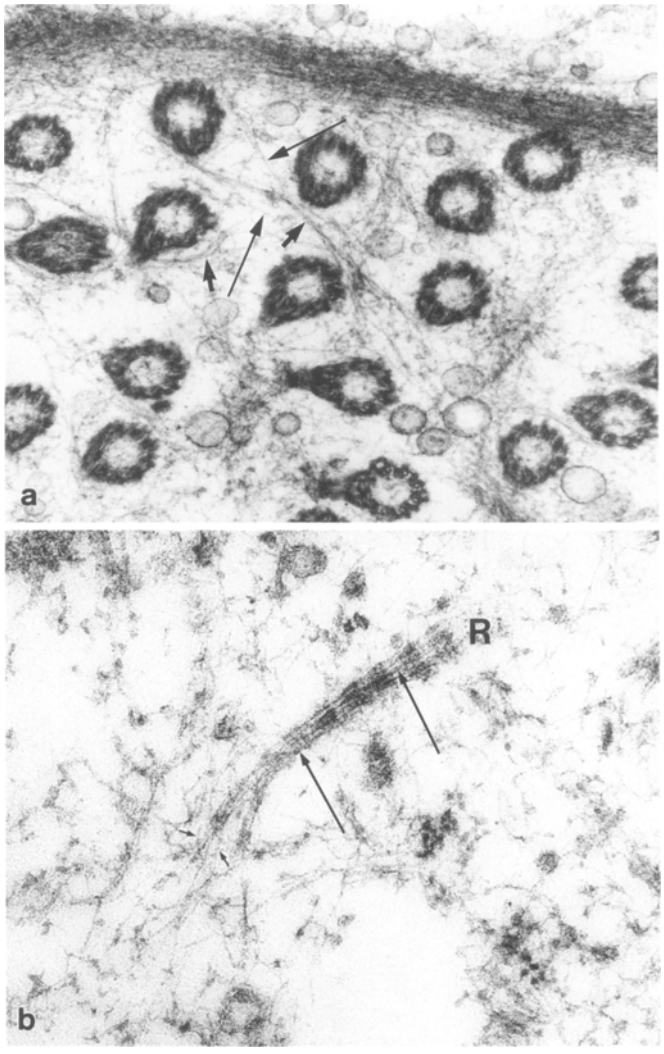

Fig. 3a. In the apical filament- *(long arrows)* and microtubule system *(short arrow)* anchored, transversely sectioned basal body of the cilia. TEM, 95040 X. **b** Pronounced striation of a longitudinally sectioned root *(R)* of a cilium. The root consists of partially transversely sectioned microtubuli *(long arrows)* and has direct contact to the cytoplasmic microtubuli *(short arrows)*. TEM, 118800 X

Some of the intermediate filaments, radiating through the whole cytoplasm from the nucleus into the cellular periphery are attached to the desmosomes (Fig. 1a, b) and hemidesmosomes (Fig. 1b). In the apical area facing the bronchial lumen a more complex connecting system exists between the cells of the bronchial epithelium, also referred to as apical terminal crest or adhesive complex. Intermediate filaments radiate not only in the desmosomes but also into the zonulae adhaerentes. Depending on the level of the section a variably thick and dense network running parallel to the surface is developed in the apical cytoplasm, which is directly associated to the adhesive complex (Fig. 2). This filamentary complex exhibits similarities to the terminal filamentary network (terminal web) of the gut epithelium.

In systematic studies a recurrent polar network system can be recognized, depending on the level of section. It

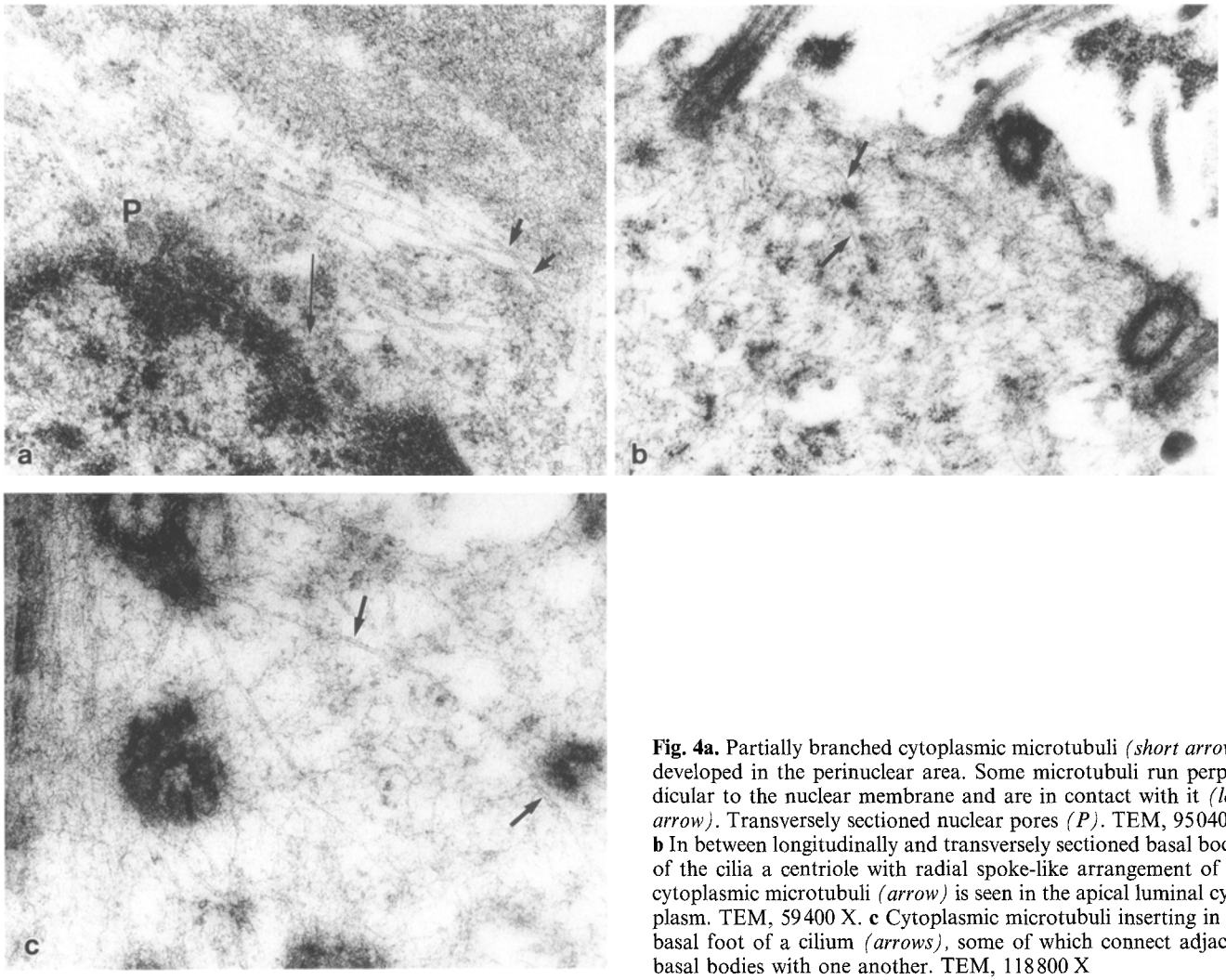

Fig. 4a. Partially branched cytoplasmic microtubuli *(short arrows)* developed in the perinuclear area. Some microtubuli run perpendicular to the nuclear membrane and are in contact with it *(long arrow)*. Transversely sectioned nuclear pores *(P)*. TEM, 95040 X. **b** In between longitudinally and transversely sectioned basal bodies of the cilia a centriole with radial spoke-like arrangement of the cytoplasmic microtubuli *(arrow)* is seen in the apical luminal cytoplasm. TEM, 59400 X. **c** Cytoplasmic microtubuli inserting in the basal foot of a cilium *(arrows)*, some of which connect adjacent basal bodies with one another. TEM, 118800 X

consists of intermediate filaments radiating out of the nucleus into the cytoplasm, and microfilaments in contact with the zonulae adhaerentes are arranged circularly in the apical cytoplasm. The filamentary structures are partly arranged in a retiform manner and partly in a parallel system so that a dense network running parallel to the surface develops in the apical cytoplasm (Fig. 2). Cytoplasmic microtubules are interspersed in this filamentary network. Some of them also run parallel to the surface entering into the adhesive complex (Fig. 2).

The anchoring elements of the cilia are interwoven in this apical filamentary network (Fig. 3a). Intermediate filaments, microtubules and microfilaments are directly associated with the individual basal bodies and roots (Fig. 3a, b). Filamentary structures and individual microtubules radiate out of this dense network into the individual microvilli (Fig. 2).

The microvilli and the cilia possess a double-contoured membrane arising from the cytoplasmic membrane. In the centre of the microvilli an electron-dense core composed of microfilaments and microtubules can be identified. These central microfilaments are formed out of the horizontal filaments running from the apical network. Some of them radiate directly into the zonulae adhaerentes.

Depending on the level of the section a variably dense system of microtubuli is evident in the ciliated epithelial cells. Some microtubules are stretched, some are curved and they can be demonstrated in all parts of the cytoplasm. They have an orderly, sometimes radial, spoke-like arrangement in the cytoplasm. They run toward the luminal cell surface and are in contact with the basal bodies of the cilia. The basal foot represents a contact area between the basal body of the cilium and the microtubules (Fig. 4a). Microtubules connecting the individual basal bodies with one another can also be demonstrated (Fig. 4a) and are seen in the microvilli reaching the apex (Fig. 2). Single or multiple electron dense zones are developed in the cytoplasm, out of which radial spoke-like microtubules emerge. These complexes are referred to as "satellites" and are located mainly in the apical luminal cytoplasm (Fig. 4b). Centrosomes with radial spoke cytoplasmic microtubules are also present where the microtubules are in contact with the outer nuclear membrane (Fig. 4c). There is direct contact between the microtubules and the intermediate filaments with organelles in between.

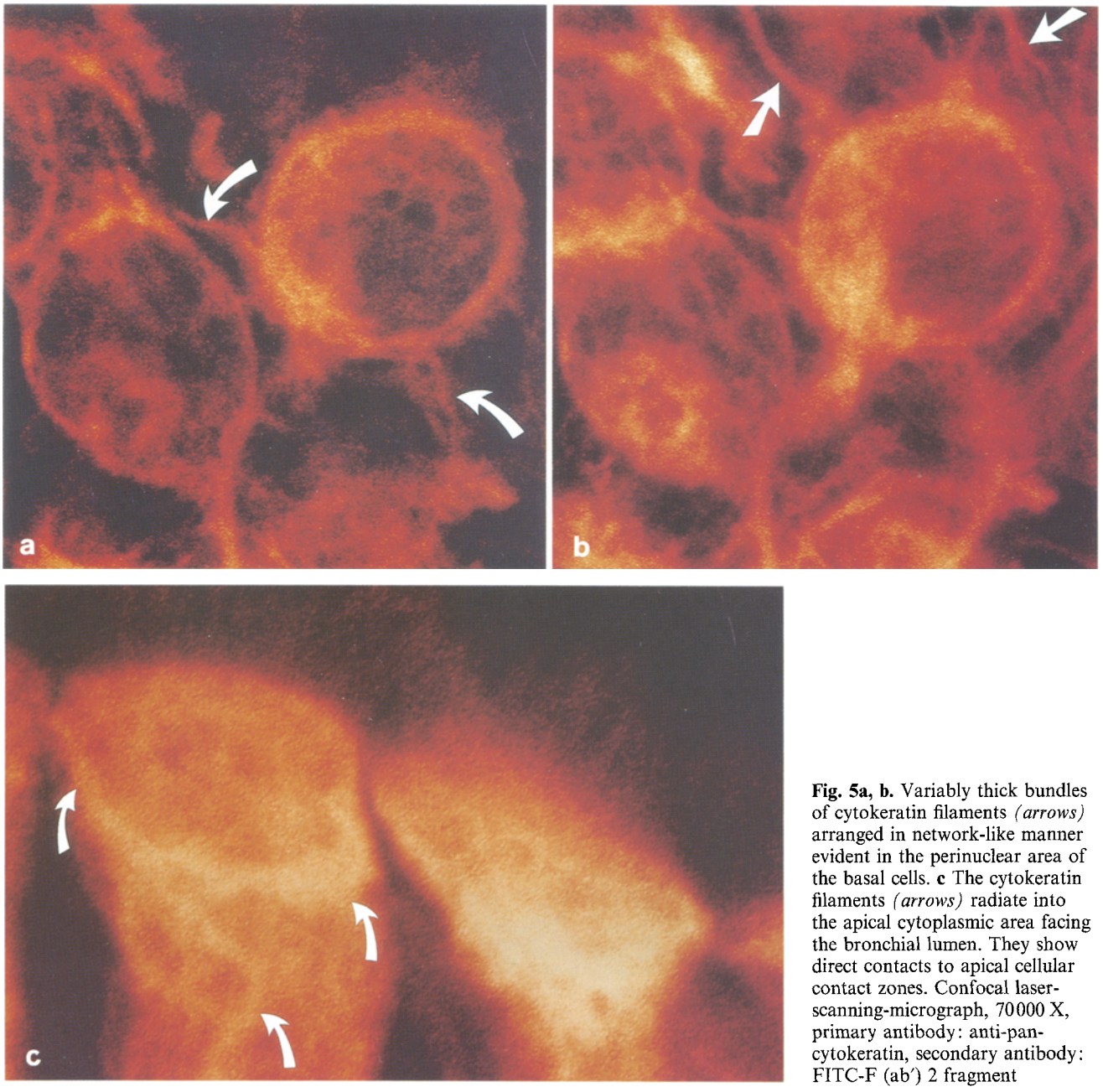

Fig. 5a, b. Variably thick bundles of cytokeratin filaments *(arrows)* arranged in network-like manner evident in the perinuclear area of the basal cells. **c** The cytokeratin filaments *(arrows)* radiate into the apical cytoplasmic area facing the bronchial lumen. They show direct contacts to apical cellular contact zones. Confocal laser-scanning-micrograph, 70000 X, primary antibody: anti-pan-cytokeratin, secondary antibody: FITC-F (ab') 2 fragment

In frozen sections the cytokeratin filaments marked by a pan-cytokeratin antibody can be identified by fluorescence microscopy and confocal laser-scanning-microscopy. The filamentary cytokeratins in association with the cellular contact zones are demonstrated by three dimensional laser electronic depiction of the cytoskeleton (Fig. 5a–c). Using a pan-cytokeratin antibody a positive reaction for the perinuclear and supranuclear intermediate filaments as well as those in the apical, filamentary system in visible. The filaments associated with the cell-contact zones are also marked by immunogold. Filamentary structures reaching to the cytoplasmic membrane and into the microvilli above the "terminal" network are also marked. A distinct marking at the points of anchorage cytoskeleton the basal bodies and the roots of the cilia and the apical filamentary system is seen (Fig. 6). Immunomarking of histological sections for SEM shows a distinct decoration especially in the apical cytoplasm. At the level of the centrioles a prominent cytokeratin reaction is evident, showing a band-like arrangement which is developed in all ciliated cells at the same level. In the vicinity of and in between the individual basal bodies of the cilia a positive antibody-reaction is visible. This reaction was not be demonstrated in the fine filamentary structures directly connecting the individual cilia with one another below the cytoplasmic membrane (Fig. 7). In the basal cells a distinct perinuclear arrangement of the silver particles is apparent. Silver deposits can be also identified from the perinuclear spaces up to the narrow cytoplasmic projections and the contact zones.

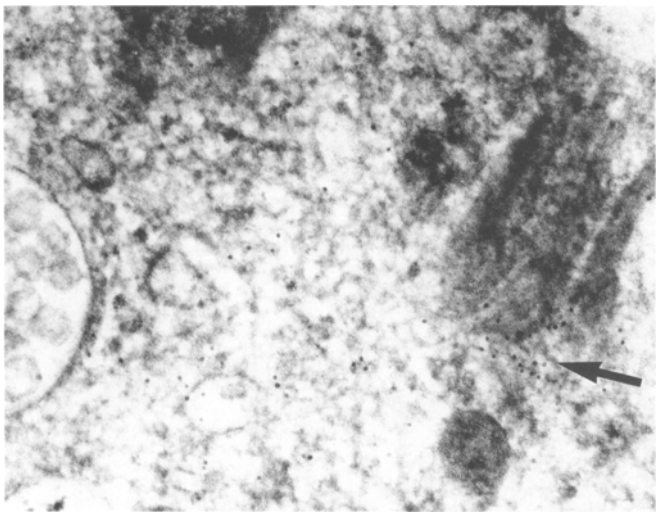

Fig. 6. Sectioning of two adjacent basal bodies. The cytokeratin filaments in the dense apical filamentary system are marked by electron dense gold. The anchoring points of the basal bodies *(arrow)* to the cytokeratin filaments are obvious. TEM, 118800 X, primary antibody: anti-pan-cytokeratin, secondary antibody: anti-goat-anti-mouse IgG gold conjugate 10 nm

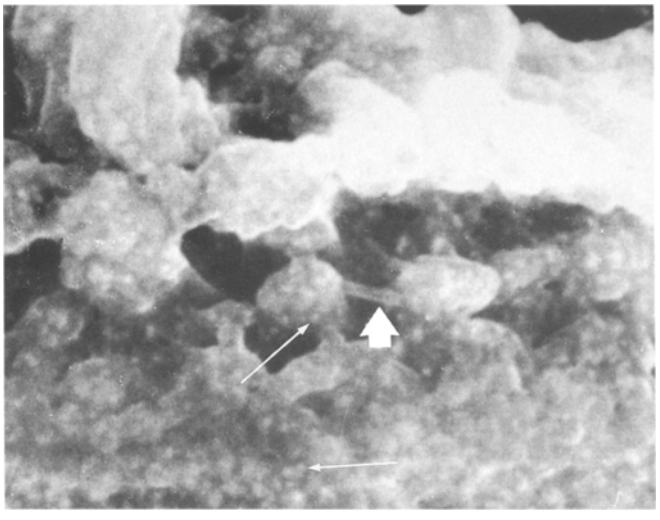

Fig. 7. Fine filamentary structures are seen *(thick arrow)* between the transversely sectioned basal bodies connecting the basal bodies with one another with a negative reaction. Silver particles *(narrow arrow)*. SEM. Immunogold marking and silver development on histological sections for scanning electron microscopy. Primary antibody: anti-pan-cytokeratin, secondary antibody: anti-goat-anti-mouse IgG gold conjugate 10 nm and silver developer, 35000 X

Discussion

Under normal conditions mucociliary transport is directed in the same direction (Messerklinger 1951; Tremble 1962). Even after injury of the ciliated epithelial mucosa the regenerating epithelial cells adapt their stroke direction to that of the surrounding cells (Proetz 1953). The exact mechanisms leading to an effective ciliary beat are not known. Our studies of the bronchial mucosa in tissue culture using human surgical and biopsy material (Philippou et al. 1993b, c) denote that co-ordinated and adequate ciliary function is possible despite interruption of the nerve supply. These findings indicate that ciliary function of the bronchial epithelium is determined by a further regulatory mechanism, independent of the nerve supply and an intra-epithelial or intracellular control mechanism must therefore be postulated.

Ultrastructural and biochemical findings (Duncan and Ramsay 1965; Gibbons and Rowe 1965; Sorokin 1968; Cordier 1975; Davies 1980; Drettner and Aust 1977; Drettner 1982; Reimer et al. 1981; Menco and Farbman 1985; Rautiainen et al. 1984) allow us to draw conclusions from the development and course of the cycle of movement of the individual cilium. However, these findings do not explain the exact mechanism of a co-ordinated and effective beat not only in the cilia of one cell but also in the entire respiratory tract.

In our light microscopic and ultrastructural studies of the cytoskeleton of the bronchial epithelium we were able to demonstrate that variably thick bundles of cytokeratin filaments are arranged around the cellular nucleus having direct contact with it. These findings, are in accordance with previous descriptions (Geuens et al. 1983; Goldman et al. 1986; Katsuma et al. 1987; Philippou et al. 1993d). We have shown, in addition that direct entrance of the filaments into the cellular adhesion complexes in the apical cell area and in the lateral and basal desmosomes and cellular contact zones and into the hemi-desmosomes of the basal cells can be demonstrated. Franke (1987) and Bologna et al. (1986) have described similar findings in immunohistochemical studies.

The filamentary network of intermediate and micro-filaments is morphologically analogous to the terminal web of the epithelium of the small intestine. The micro-filaments emanating horizontally and circularly from the zonula adhaerens correspond with the zonula adhaerens-associated filaments in the epithelium of the small intestine described by Hull and Staehlin (1979). Demonstration of variably thick interwoven bundles of cytokeratin filaments reaching from the nucleus to the cytoplasmic membrane, to the cilia and microvilli as well as the cellular contact zones provides evidence that there is a function for the system. The structure and arrangement of the cytokeratin system suggest that the cytokeratin filaments are a type of information carrier between the nucleus and the periphery of the cell. Indirect connections of the nucleus to the cilia and microvilli via the cytokeratin filaments and the entrance of the cytokeratin filaments into the cellular contact zones, are indications that an exchange of information as mechanical stimuli is possible within and between cells. Our ultrastructural studies of the cytoskeleton of the ciliated epithelial cells demonstrated a dense, radial spoke network of microtubules and connection to the cellular contact zones was seen. The microtubules system plays a role in intracytoplasmic transport, in cell division and in anchorage of receptors at the cell surface (Brinkley 1985) and the demonstration of a well-developed cytoplasmic micro-

tubules system with direct contact to the cilia and microvilli allows us to postulate that this system has a special function in the cilated cells as well as in the function of the individual cilia and microvilli.

The finding that microtubules reach into the tip of each microvillus is in accordance with reports of Brinkley (1985), who mentions that the microtubules serve as an anchorage for receptors at the cell surface, indicating that receptors are present at the surface of the microvillus. The microtubules inserting into the basal bodies of the cilia, some of which run parallel to the cell surface, might play a significant role in the co-ordination of the ciliary beat of a cell. The fact that the microtubules as well as the intermediate and microfilaments enter the cellular contact zones, supports the idea that co-ordination of the ciliary beat between adjacent cells is a special function of this system.

The results of this ultrastructural study allows a presumption that the co-ordination and effectiveness of the ciliary beat in the individual cell is controlled by the cytoskeleton, in part especially by the cytokeratin filamentary system, the cytoplasmic microtubule system and the microfilaments.

From these morphological findings it is reasonable to assume that the synchronisation of the ciliary beat with adjacent cells is influenced by the cytokeratin filamentary system with participation of the microfilaments and microtubules. During development of the beat of the cilia tractive power arises in the area of the apical filamentary system anchored basal body of the cilium, which is transferred to the neighboring basal body or to the cellular contact zones and from here to the adjacent cells. These forces transferred by the filamentary system can be effective, as mechanical stimuli on the respective cilium and on the adjacent cells, and may thus function as an initial trigger and co-ordinator of the ciliary beat. Furthermore, it is conceivable that rapid transport of ions via the cytoplasmic microtubule system could cause local changes in potential or transport of substances in the form of chemical stimuli co-ordinating the ciliary beat. In addition, the close topographical relationship between the intermediate filaments, the cytoplasmic microtubules and the cellular organelles infers that the cytoskeleton has not only a static but also a dynamic function.

Our findings demonstrate that in addition to the influence of the nerve supply the function of the bronchial epithelium is determined by further intracellular and intra-epithelial regulation which is controlled by the cytoskeleton of the bronchial epithelium. This dual regulation permits a response of the system towards changing environmental factors.

Morphological findings alone can not demonstrate a relationship between the structure and function of the individual components of the cytoskeleton and their influence on ciliary function. There are currently no methods which allow us to establish this relationship in vivo, thus we must depend on the postulation and confirmation of possible hypotheses.

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