16HBE14o- Human Bronchial Epithelial Cell Layers Express P-Glycoprotein, Lung Resistance-Related Protein, and Caveolin-1

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Purpose. To study the expression of P-glycoprotein (P-gp), lung resistance-related protein (LRP), and caveolin-1 (cav-1) in the human bronchial epithelial cell line *16HBE14o-*.

Methods. The presence of P-gp, LRP, and cav-1 in *16HBE14o-* cell layers was evaluated using immunocytochemical staining and visualization with confocal laser scanning microscopy (CLSM). Functionality of P-gp was determined by bidirectional transport of rhodamine-123 with and without a P-gp inhibitor, verapamil. Caveolae were visualized using transmission electron microscopy (TEM). Flux of fluorescein-Na was also studied as a paracellular transport marker.

Results. Immunocytochemical staining showed expression of P-gp localized at the apical membrane of *16HBE14o-* cell layers. The flux of rhodamine 123 across cell layers exhibited a greater P_{app} value for the secretory (i.e., basolateral-to-apical) direction. This asymmetry disappeared in the presence of verapamil. CLSM provided evidence for the expression of LRP and cav-1. TEM further showed typically shaped caveolae at the apical and basolateral membranes.

Conclusion. Cell layers of *16HBE14o-* express drug transport systems that are also present in the human bronchus *in vivo*, indicating that the *16HBE14o-* cell line may be a suitable candidate for an *in vitro* model for mechanistic studies of drug transport processes involved in the smaller airways.

KEY WORDS: caveolae; cell culture; airway epithelium; pulmonary absorption.

INTRODUCTION

The lung is considered an attractive route for systemic drug delivery because some peptides and protein drugs, which show poor systematic bioavailability via the oral route, apparently achieve excellent bioavailability when delivered by inhalation as pharmaceutical aerosols (1). In addition to targeting to the deep lung for systemic absorption, aerosols are also routinely delivered to the bronchial regions of the airways for the treatment of acute or chronic lung diseases (e.g., asthma or cystic fibrosis).

Studies using epithelial cell culture models that resemble the native barrier have made significant impact on the progress of biopharmaceutical sciences in recent years. In this context, protocols have been developed for the isolation and cultivation of primary respiratory epithelial cells, either of human or of animal origin (2,3). Additionally, cancer cell lines have been screened for their suitability to serve as *in vitro* models (4,5), and new airway epithelial cell lines that preserve the differentiated phenotype were obtained by immortalization (6). However, primary culture is costly and requires a large number of laboratory animals, whereas cancer cells often lack the specific phenotype.

The cell line *16HBE14o-* was generated by transformation of normal bronchial epithelial cells obtained from a 1-year-old heart–lung transplant patient. Transformation was accomplished with SV40 large T antigen using the replication-defective pSVori$^-$ plasmid (6). The use of *16HBE14o-* cell layers for the study of bronchial epithelial transport properties of pharmaceutically relevant substances has recently been reported (7,8). In particular, our laboratories very recently reported the influence of apical fluid volume on the formation of a barrier with functional tight junctions in *16HBE14o-* (9). However, information on functionality and expression levels of drug transport-related proteins in this cell line is scarce, although such data would be very important in the context of drug development and delivery to the lungs.

The P-glycoprotein (P-gp) is a 170-kDa protein located in the plasma membrane, extruding a range of hydrophobic drugs and natural compounds from cells against the concentration gradient. Recently, it has been recognized that P-gp shows a broad overlap in substrate and inhibitor specifities with the metabolizing enzyme CYP3A4 (10). P-gp is a member of the ABC (ATP-binding cassette) superfamily of transporters, which are coded in humans by the *mdr* genes. P-gp has been shown to be expressed in several human tissues, especially in organs with an excretory function (colon, kidney), capillary cells of the brain and testis, and the normal human bronchus (11,12).

The lung resistance-related protein (LRP) has been identified as the MVP (major vault protein), the main component of vaults (13). Vaults are oval-shaped cytoplasmic particles with a size of ~65 × 35 nm. With a molecular mass of about 13 MDa they are the largest ribonucleoprotein complexes known (three times the size of a ribosome) (14). Mammalian vaults are composed of multiple copies of three proteins—the cap proteins [240 kDa (TEP1), 193 kDa (VPARP)], the barrel protein [104 kDa (LRP)], and unique untranslated RNA species (15). Higher levels of vaults (~100,000/cell) are found in tissues (e.g., kidney, colon, and bronchus) that are chronically exposed to elevated levels of xenobiotics, in metabolically active tissue (e.g., adrenal cortex), and in macrophages (16,17). By the fluorescence recovery after photobleaching (FRAP) technique, vaults were shown to be very mobile (18). Several studies have implicated vaults in nucleocytoplasmic trafficking and transport of different substrates including steroid hormone receptors and ribosomes (15). In addition,

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vaults may mediate multidrug resistance (MDR) by the compartmentalization of drugs away from intracellular drug targets (19). Through exocytotic vesicles or pump molecules such as P-gp, MRP1, and BCRP, the drugs may be extruded from the cells (15).

Caveolae are flask-shaped invaginations present in the plasma membrane of many cell types. They are abundant in type I pneumocytes and capillary endothelial cells of the lung, and their presence has recently been confirmed in native and primary cultured human bronchial epithelial cells (20,21). Caveolae have been implicated in potocytosis, transcytosis, and cell signaling. Recent work has confirmed that caveolae are directly involved in the internalization of membrane compounds (e.g., glycosphingolipids), extracellular ligands (e.g., folic acid, albumin), bacterial toxins (cholera toxin, tetanus toxin), and nonenveloped viruses (Simian virus 40, polyoma virus) (22–24). FRAP analysis has shown that some caveolae may be stationary and held in place by the cortical actin cytoskeleton underlying the plasma membrane (25). Unlike clathrin-mediated endocytosis, internalization and subsequent intracellular trafficking through caveolae is a triggered event that involves complex signaling (24). The structural proteins of caveolae are the caveolins (cav-1, cav-2, and cav-3). Cav-1 is a 21- to 24 kDa protein that exists in oligomers comprised of 14–16 monomers. These caveolin oligomers were shown to interact with each other to form caveolae-like structures *in vitro* (26). In addition, caveolins are present in the *trans*-Golgi network and in a newly discovered organelle called the "caveosome" (24).

In this study, we explored the expression profiles of P-gp, LRP, and cav-1 in *16HBE14o-* human bronchial epithelial cell layers to determine the suitability of the cell line as an *in vitro* small airway model for transport/metabolism studies.

MATERIALS AND METHODS

Materials

Rhodamine 123, fluorescein-Na, doxorubicin-HCl, verapamil, tissue culture media, and reagents were all obtained from Sigma (Deisenhofen, Germany). Tissue culture plastics from Greiner (Frickenhausen, Germany) and Transwell Clear inserts (12 mm in diameter, pore size 0.4 μm) from Corning Costar (Bodenheim, Germany) were used. Rabbit polyclonal cav-1 antibody was obtained from BD Transduction Laboratories (Heidelberg, Germany), mouse monoclonal P-gp antibody (clone F4) was purchased from Sigma, mouse monoclonal LRP-56 antibody from Sanbio (Beutelsbach, Germany), and rabbit polyclonal anti proSP-C antibody from Chemicon (Hofheim, Germany). An FITC-labeled mouse monoclonal (clone Ks 18.27) cytokeratin 18 antibody was purchased at Bender MedSystems (Vienna, Austria). FITC-labeled goat antimouse $F(ab')_2$ fragment or swine anti-rabbit $F(ab')_2$ fragments (both DAKO, Hamburg, Germany) were used as secondary antibodies. FluorSave antifade medium was obtained from Calbiochem (Bad Soden, Germany), and epoxy-resin EMbed 812 from EMS (Fort Washington, PA).

Cell Line and Culture Conditions

Passage 2.47 to 2.87 of *16HBE14o-* cells, a gift from Dr. Dieter C. Gruenert (Department of Medicine, University of Vermont, Burlington, VT), were used in this study. Cells were seeded onto Transwell Clear permeable filter inserts at a density of 10^5 cells/cm^2. These cells were grown under liquid-covered conditions (i.e., 500 μl apically, 1500 μl basolaterally) in Eagle's minimum essential medium (EMEM) supplemented with 10% fetal calf serum, 0.1 mM nonessential amino acids, 2 mM L-glutamine, 100 μg/ml streptomycin, and 100 U/ml penicillin G, at 37°C in a 5% CO_2 incubator. The culture medium was changed daily. Preliminary studies showed no significant influence of coating the filters either with fibronectin/collagen or Vitrogen-100 on the transepithelial electrical resistance (TEER) over 21 days in culture (data not shown); thus, we plated cells onto bare Clearwells. To assure the quality of the cell line, immunocytochemical staining studies were performed. Antibodies against the epithelial marker cytokeratin 18 and the lung-specific prosurfactant protein C were used, revealing that >99% of cells were positive for both markers by FACS analysis (data not shown). TEER measurements and light microscopy established the optimal seeding density at 10^5 cells/cm^2 (9).

Immunocytochemical Staining

Rabbit polyclonal cav-1 antibody, mouse monoclonal P-gp antibody (clone F4), and mouse monoclonal LRP-56 antibody were all diluted 1:100 in phosphate-buffered saline (PBS) containing 1% (w/v) BSA. Mouse IgG1κ (Sigma) was used as an isotypic control. Cell layers were stained 1 week after seeding when TEER values peaked (671 ± 133 Ω•cm^2, $n = 60$) (9). Cells were fixed for 10 min with 2% (w/v) paraformaldehyde (PFA) and blocked for 10 min in 50 mM NH_4Cl, followed by permeabilization for 8 min with 0.1% (w/v) Triton X-100. After 60 min of incubation with 100 μl dilution of one of the primary antibodies, the cell layers were washed three times with PBS before incubation with 100 μl of a 1:100 dilution of a FITC-labeled goat antimouse $F(ab')_2$ fragment or swine antirabbit $F(ab')_2$ fragment in PBS containing 1% (w/v) BSA. Propidium iodide, 1 μg/ml, was then added for counterstaining of cell nuclei. After 30 min incubation, the specimens were washed three times with PBS and embedded in FluorSave antifade medium. Images were obtained by a confocal laser scanning microscope (MRC-1024, Biorad, München, Germany) with the instrument settings adjusted so that no positive signal was observed in the channel corresponding to green fluorescence of the isotypic controls.

Intracellular Doxorubicin Distribution

The analysis of the intracellular doxorubicin (DOX) distribution was carried out by confocal laser scanning microscopy by taking advantage of the intrinsic fluorescence of the drug. The observations were performed on cell layers grown for 1 week on Transwell Clear filter inserts. The cell layers were incubated on both sides with 2 μM DOX in culture medium for either 10 min or 30 min at 37°C. In order to detect autofluorescence, the untreated cells were also observed under the same instrumental settings. In addition, to detect the response of intracellular LRP to the DOX exposure, cells were fixed with PFA and incubated with the LRP-56 mAb as described above directly after the DOX solution was removed and the cells washed twice with PBS. To see colocalization, an FITC-labeled goat antimouse secondary antibody was used.

Transport Studies

Transport experiments were conducted using *16HBE14o-* cells from two different passages cultured for 1 week. Both sides of cell layers were washed twice with pre-equilibrated bicarbonated Krebs–Ringer solution (KRB, 15 mM HEPES (*N*-[2-hydroxyethyl]piperazine-*N'*-[2-ethanesulfonic acid]), 116.4 mM NaCl, 5.4 mM KCl, 0.78 mM NaH_2PO_4, 25 mM $NaHCO_3$, 1.8 mM $CaCl_2$, 0.81 mM $MgSO_4$, 5.55 mM glucose, pH 7.4). Clearwell-grown cell layers were then placed in new cluster plates containing 1.5 ml per well of KRB prewarmed to 37°C. In P-gp inhibition experiments, verapamil at 50 μM was present in donor and receiver solutions to ensure a continued inhibition of the P-gp efflux pump. After 60 min of equilibration with KRB, transport of the known P-gp substrate rhodamine-123 (Rh123, M_w 380.8) or fluorescein-Na (FluNa, M_w 376.3, as a paracellular transport marker) was initiated by replacing the fluid in the donor chamber with KRB containing either 50 μM Rh123 or 50 μM FluNa. The apical and basolateral fluid volumes were 0.52 and 1.5 ml, respectively. The initial concentration in the donor fluid was assayed by drawing a 20 μl sample immediately after the initiation of flux measurements. The cell layers were agitated by an orbital shaker at a constant stirring rate (100 rpm) at 37°C during transport experiments. Two-hundred-microliter samples were drawn serially from the receiver compartment at 15, 30, 45, 60, 75, and 90 min. After each sampling, fresh transport buffer of an equal volume was returned to the receiver side to maintain a constant volume. At the end of the transport experiment, a 20 μl sample was drawn from the donor fluid and assayed for its activity. Each experiment was performed, employing a total of 12 cell layers from two different passages. In order to assess the integrity of cell layers during the flux experiment, TEER was measured before and after each transport experiment.

Flux (J) was determined from steady-state appearance rates of each drug in receiver fluid. The apparent permeability coefficient, P_{app}, is calculated according to the equation $P_{app} = J/(A \bullet C_i)$, where C_i was the initial concentration of the drug under investigation in the donor fluid and A the nominal surface area of cell layers (1.13 cm^2) utilized in this study. Fluorescence of samples was analyzed in 96-well plates using a fluorescence plate reader (Cytofluor II, PerSeptive Biosystems, Wiesbaden, Germany) at excitation and emission wavelengths of 485 and 530 nm, respectively. These samples were diluted with KRB, where appropriate.

Electron Microscopy

16HBE14o- cell layers cultured on Transwell Clear filters were washed with PBS and fixed in a mixture of 1% (w/v) paraformaldehyde and 1% (w/v) glutaraldehyde in 0.1 M phosphate buffer for about 12 h at 4°C. Washed cells were treated with 2% (w/v) osmium tetroxide and 1.5% (w/v) potassium ferrocyanide ($K_4[Fe(CN)_6]$) in 0.1 M phosphate buffer for 4 h at room temperature. Several washing steps with buffer and water removed nonbound salts before *en bloc* contrasting for 45 min in aqueous 2% (w/v) uranyl acetate was performed. These cell layers were then washed with water and dehydrated by increasing concentrations of ethanol [35, 50, 75, and 90% (v/v), each step for 10 min]. Infiltration with the epoxy resin EMbed 812 was performed by serial incubation with hydroxypropyl methacrylate (HPMA) [90, 95, and 97% (w/v), 15 min each] as an intermedium. These processed cell layers on Clearwell filters were cut into smaller pieces and infiltrated with mixtures of HPMA and EMbed (2:1, 1:1, each for 15 min, and 1:2 for 30 min) and pure EMbed (12 h). Finally, filters were embedded in fresh EMbed and polymerized at 60°C.

Ultrathin sections (60–80 nm thick) were taken perpendicular to the filter surface using an ultramicrotome (Leica, Bensheim, Germany). After staining with uranyl acetate and lead citrate for more contrast, sections were observed and photographed using a transmission electron microscope (EM10C, Zeiss, Oberkochen, Germany) at 60 or 80 kV.

Statistical Analysis

Results are expressed as mean ± SD. Significance ($p < 0.05$) of differences in the P_{app} values from several ($n \geq 3$) data groups were determined by one-way analysis of variance (ANOVA) followed by Neumann–Keuls–Student *post-hoc* tests.

RESULTS

Immunocytochemical Staining

Immunocytochemical staining, performed after 1 week culture of *16HBE14o-* cells, showed strong expression of P-gp in the apical, but not basolateral, plasma membrane (Fig. 1A). The P-gp signal was strongly localized along the apical membrane when cross sections of cell layers were examined (Fig. 1B). Staining for LRP resulted in a clear signal with a punctate staining pattern typical for intracellular vesicles or similar substructures (Fig. 2A). The staining pattern observed for cav-1 by CLSM clearly and reproducibly showed organization along the cell membrane. Additional cav-1–positive intracellular objects are of an irregular vesicular shape (Fig. 2B). Double-staining experiments to investigate a possible association of caveolae and LRP did not show any colocalization (data not shown).

Intracellular Doxorubicin Distribution

Confocal microscopy revealed a time-dependent distribution of intracellular DOX (Fig. 3). After 10 min of treatment (Fig. 3, left panel), *16HBE14o-* cells showed a low total fluorescence intensity that was mainly concentrated in vesicular structures. After 30 min of treatment, higher DOX amounts were already detectable in the nuclei of *16HBE14o-* cells (Fig. 3, right panel). The costaining with the LRP-56 antibody showed that most cells were LRP-positive after 10 min of DOX treatment, showing the punctate pattern similar to that observed in untreated cells (Fig. 3C). After 30 min of treatment, no more staining in vesicular structures could be found. Instead, the LRP signal was very strong in the perinuclear region, and there was also diffuse labeling in the cytoplasm (Fig. 3D). It should be noted that the orange-yellow punctate signal observed after 10 min of treatment is strongly

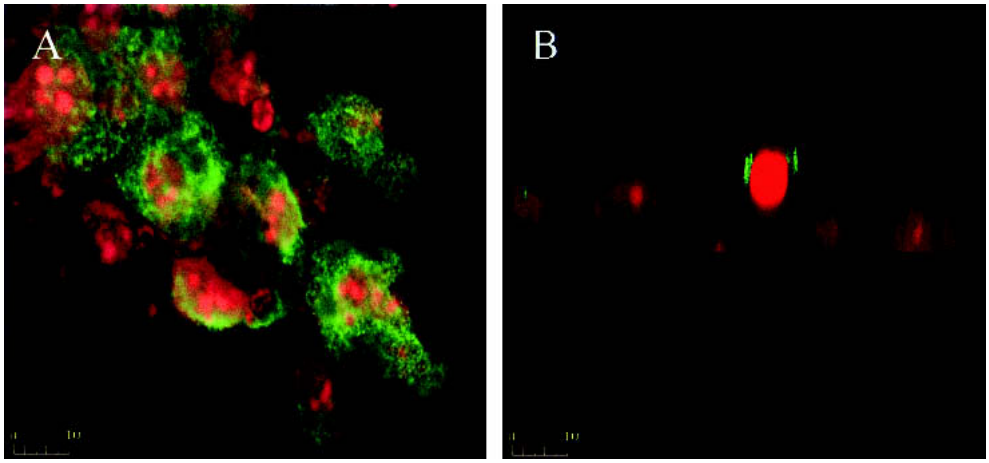

Fig. 1. Immunolabeling of P-gp in Clearwell-grown *16HBE14o-* cell layers after 1 week. Cells were seeded at a density of 10^5 cells/cm^2 and cultured under liquid-covered culture conditions. Staining for P-glycoprotein (green) is shown in A using CLSM. A vertical cross section is given in B. Nuclei were counterstained with propidium iodide (red) in both panels. *Scale bars* represents 1 μm.

indicative of a colocalization of DOX and LRP in vesicular structures.

Transport Studies

A significantly ($p < 0.05$) higher P_{app} ($4.46 \pm 0.28 \times 10^{-6}$ cm•s^{-1}, $n = 12$) of Rh123 was observed in the secretory (i.e., basolateral-to-apical) direction (Table I), which was significantly reduced by 63% in the presence of verapamil. Permeability of Rh123 in the apical-to-basolateral direction was $1.52 \pm 0.24 \times 10^{-6}$ cm•s^{-1}, $n = 12$, which was not significantly altered in the presence of verapamil. The secretion/absorption-ratio for *16HBE14o-* cell layers was 2.95, although it was 4.80 in transport experiments carried out with 3-week-old *Caco-2* cells under the same conditions (Table I).

The P_{app} value of the paracellularly transported FluNa across *16HBE14o-* cell layers showed no significant ($p < 0.05$) changes after addition of verapamil and was ~60% of P_{app} for Rh123 in the apical-to-basolateral direction. The permeability of FluNa across 3-week-old *Caco-2* monolayers was approximately 2.8-fold lower, although the TEER values of *16HBE14o-* and *Caco-2* cell layers were comparable.

Electron Microscopy

16HBE14o- cells grown on Transwell Clear filters formed a multilayer usually one to five cells in thickness with well-developed cell–cell contacts (compare also with ref. 9) and microvilli (Fig. 4A). Apical and basolateral plasma membranes showed caveolae-like invaginations (i.e., omega-shaped) in all cells including those of the intermediate layers (Fig. 4A,B).

DISCUSSION

In the present study, we have evaluated the expression of drug transporters and transport-related proteins in the human bronchial epithelial cell line *16HBE14o-,* which has previously been suggested as a model for drug transport studies

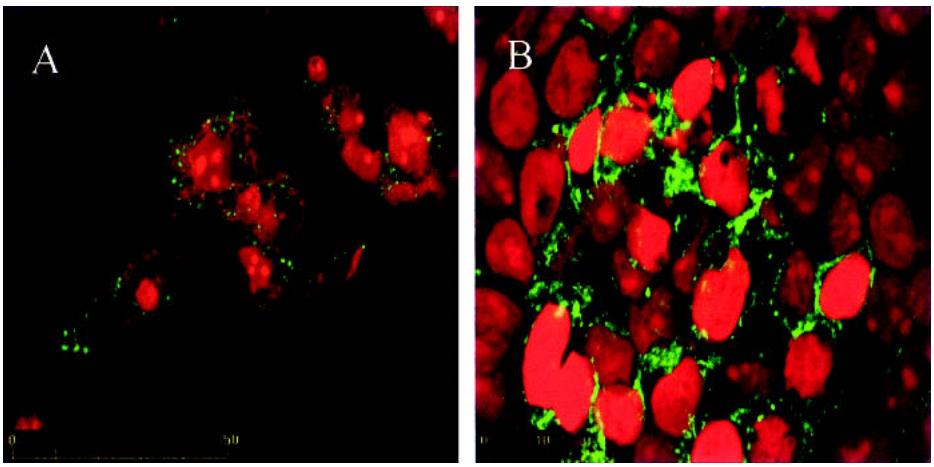

Fig. 2. Immunolabeling of LRP and caveolin-1 in *16HBE14o-* cells grown on Clearwell filters. Cell layers cultured for 1 week were stained using antibodies against LRP (A) or caveolin-1 (B) in combination with an FITC-labeled secondary antibody (green). Nuclei were counterstained with propidium iodide (red). *Scale bars* represents 1 μm.

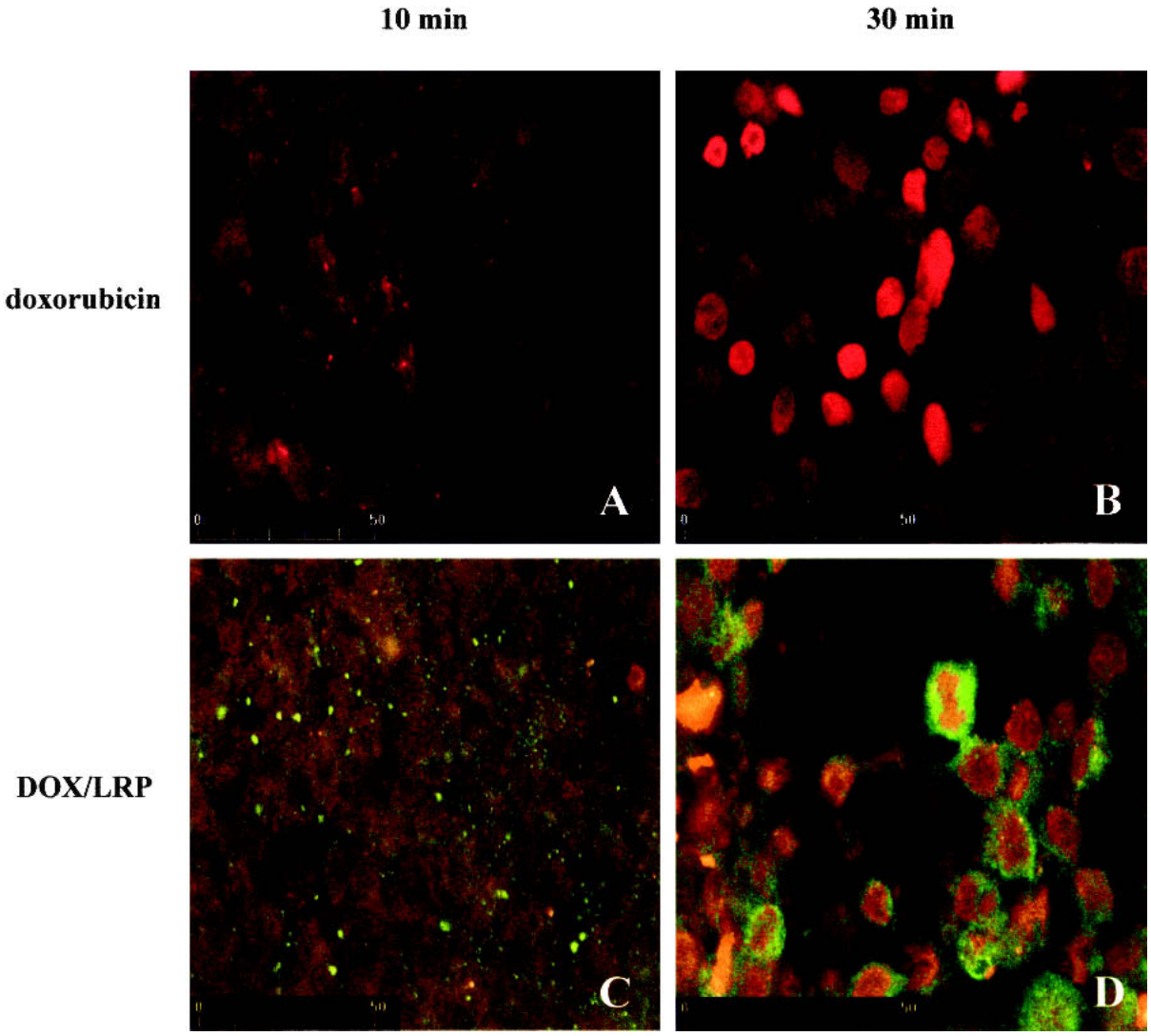

Fig. 3. Intracellular distribution of doxorubicin (DOX) and colocalization between DOX and LRP in *16HBE14o-* cells grown on Clearwell filters. Cell layers cultured for 1 week were incubated for 10 min and 30 min with DOX (red, top panel) or incubated with DOX and subsequently stained using an antibody against LRP (bottom panel) in combination with a FITC-labeled secondary antibody (green). *Scale bars* represents 1 μm.

(7,8). In addition to the ability to form a well-polarized cell layer with functional tight junctions (9), *16HBE14o-* cells were found to express P-glycoprotein, lung resistance-related protein, and caveolin-1.

The expression of MDR1 P-gp 170 was shown by immunocytochemical staining, and P-gp functionality was confirmed by the observed net secretion of rhodamine 123. The activity, based on net Rh123 secretion, was ~60% of that

Table I. Comparative P_{app} Values of Permeation Markers across *16HBE14o-* and *Caco-2* Cell Layers Cultured on Transwell Clear Filter Inserts

		P_{app} [$\times 10^{-6}$ cm · s^{-1}][a]			
		16HBE14o-		*Caco-2*	
Solutes	±Verapamil	Absorption	Secretion	Absorption	Secretion
Rhodamine 123	–	1.52 ± 0.24	4.48 ± 0.28*	0.49 ± 0.19	2.35 ± 0.15[b]
Rhodamine 123	+	1.43 ± 0.09	1.47 ± 0.11	ND	ND
Fluorescein-Na	–	0.85 ± 0.03	0.82 ± 0.04	0.26 ± 0.07	0.34 ± 0.01
Fluorescein-Na	+	0.85 ± 0.03	0.84 ± 0.02	ND	ND

[a] Mean ± S.D. ($n = 12$).
[b] Significantly ($p < 0.05$) from absorptive direction.

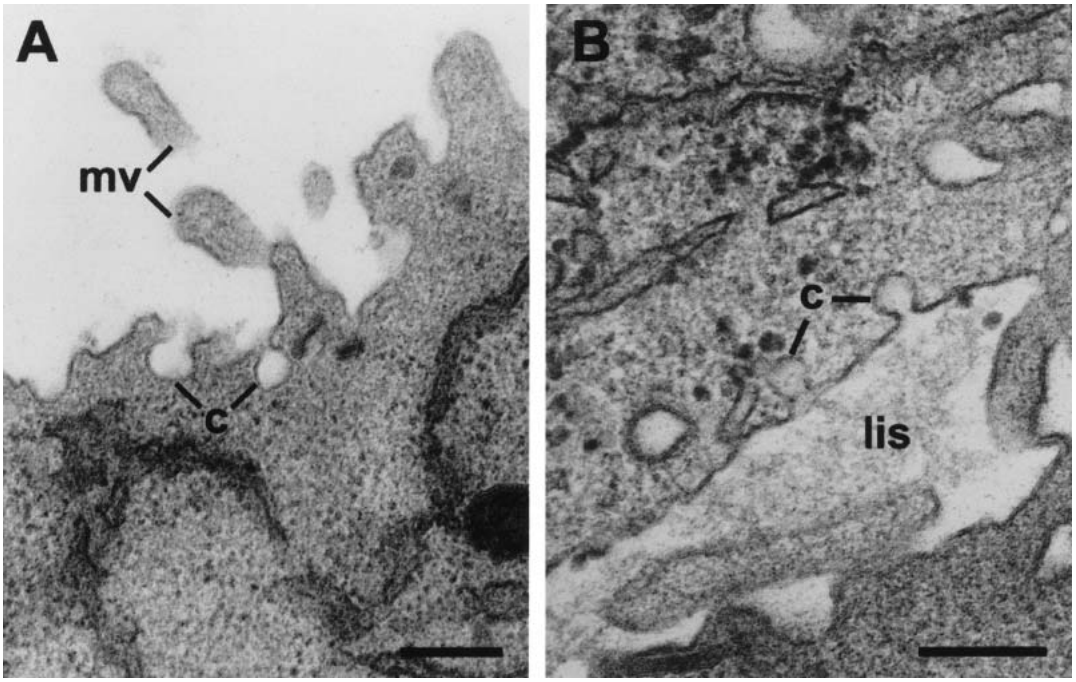

Fig. 4. Transmission electron microscopy showing the cross sections of *16HBE14o-* cells grown on Transwell Clear filters for 1 week. (A) Apical regions exhibiting microvilli (*mv*). The apical plasma membrane frequently was invaginated by caveolae-like structures (*c*). (B) Basolateral membranes of two cells were separated by the lateral intercellular space (*lis*). The cell on the right displays two typical omega-shaped caveolae-like membrane structures (*c*). Bars = 0.2 μm.

found in *Caco-2* cells and only ~25% of that reported for the *Calu-3* bronchial adenocarcinoma cell line (27).

By immunocytochemical staining, we also confirmed LRP expression. This protein has been found to be overexpressed in certain non-P-gp phenotypes of multidrug-resistant cell lines (28). In healthy cells, LRP may protect the nucleus from accumulating toxic compounds (23). However, the exact roles of LRP in most cells are still elusive, although expression of LRP was reported for native human bronchial tissue (24). Our current findings indicate that *16HBE14o-* cells may be a reasonably good model to study the putative mechanisms of LRP function in bronchial epithelial cells.

Caveolin-1 was found to be localized in vesicular structures and along the intercellular junctions of *16HBE14o-* cells by CLSM. Additionally, TEM studies showed the cell membrane localization of typical omega-shaped structures known as caveolae. However, the *Calu-1* cell line exhibited increased expression of cav-1 and -2 compared to those in native and primary cultured bronchial epithelial cells (21). It should be pointed out that in order to investigate the role of caveolin and caveolae as active moieties in macromolecular drug transport (23), cells exhibiting a confluent layer with functional tight junctions are mandatory. Because *Calu-1* cells do not form a functional polarized epithelial barrier, *16HBE14o-* is the only available cell line to study caveolae/caveolin in the context of macromolecular transport across the bronchial epithelial barrier.

In summary, the *16HBE14o-* cell line appears to possess many similarities to the native bronchial epithelium and thus may provide a very useful model for mechanistic studies of regulation of transport function of bronchial epithelium. Rapid screening of transport properties of novel drugs targeted for systemic absorption via airways or for local action at the smaller airways can be expected to be feasible using the *16HBE14o-* model.

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