Induction of vacuolar apical compartments in the Caco-2 intestinal epithelial cell line

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Summary

Complete disruption of the microtubular network by colchicine or nocodazole in Caco-2 intestinal epithelial cells results in the appearance of basolateral microvilli and brush border-containing intracellular vacuoles (vacuolar apical compartment: VAC). These vacuoles are surrounded by a terminal web, express apical markers and exclude basolateral markers. The vacuoles do not originate from internalized apical or basolateral plasma membrane and their development is blocked by protein synthesis inhibitors, suggesting that they are newly synthesized. After removal of the microtubule inhibitors, VACs are usually degraded and/or released into the lateral intercellular space. Rarely was fusion with the apical membrane observed. These experiments support a role for microtubules in the biogenesis of the apical surface and indicate that, under some conditions, apical plasma membrane assembly may occur in the cytoplasm, as observed in some human pathological states.

Key words: VAC, Davidson's disease, epithelial polarity, Caco-2 cells.

Introduction

Transporting epithelial cells exhibit two distinct plasma membrane domains, apical and basolateral, separated by tight junctions. The polarized surface distribution of transporting systems between these two surfaces underlies the many vectorial functions of epithelial cells (Simons and Fuller, 1985; Rodriguez-Boulan and Nelson, 1989). Considerable advances have been recently made on the biogenesis of epithelial cell polarity. In the model kidney-derived cell line MDCK, the biosynthetic pathways of apical and basolateral proteins appear to diverge in the trans Golgi network (intracellular sorting; Griffiths and Simons, 1986; Matlin and Simons, 1984; Misek et al. 1984; Rindler et al. 1984). Consistent with these observations, two distinct transport vesicle populations have been identified that are thought to mediate the delivery of plasma membrane proteins to the apical and basolateral domains (Wandinger-Ness et al. 1990). On the other hand, in other systems, such as intestinal and liver epithelial cells (including the human colon carcinoma cell line Caco-2), sorting of many proteins takes place at the basolateral surface (Massey et al. 1987; Bartles et al. 1987; Le Bivic et al. 1990; Matter et al. 1990).

The Caco-2 cell line undergoes a spontaneous enterocytic differentiation upon in vitro culture. These cells express a well-developed brush border and many hydrolases specific for the small intestine or the fetal colon (Pinto et al. 1983; Chantret et al. 1988). Recent work explored the role of microtubules in targeting processes in Caco-2 cells. Nocodazole treatment was found to retard both direct and indirect apical pathways with no effect on the final distribution of apical or basolateral plasma membrane proteins (Matter et al. 1990) whereas colchicine treatment led to a completely non-polarized delivery of some apical markers (Gilbert et al. 1991). In vivo, colchicine treatment is known to interfere with the apical pathway (Quaroni et al. 1979; Achler et al. 1989) and to induce ultrastructural modifications like basolateral microvilli and intracytoplasmic brush border vacuoles (Pavelka et al. 1983; Achler et al. 1989). A familial enteropathy, microvillus inclusion disease or Davidson's disease, is characterized by a highly reduced apical brush border and the appearance of large intracellular vacuoles with inwardly facing microvilli in enterocytes (Cutz et al. 1989; Davidson et al. 1978). Attempts to obtain an in vitro model for this disease were made using primary cultures of human fetal intestine but only partial results were reported (Carruthers et al. 1986).

We report here that microtubule-disrupting agents induce the appearance of basolateral brush border and apical-like intracellular vacuoles in Caco-2 monolayers. Using laser scanning confocal microscopy and electron microscopy, we investigated the formation and origin of these vacuoles and their behavior upon removal of the microtubule inhibitors. We discuss these observations as a possible in vitro model for Davidson's disease.

Materials and methods

Reagents

Cell culture reagents were purchased from Gibco Laboratories (Grand Island, NY). Sulfosuccinimidyl 6-(biotinamido) hexanone (NHS-LC-biotin) and Texas red(TR)-avidin were from Pierce (Rockford, IL). Fluorescein (FITC)-conjugated goat anti-
mouse or anti-rabbit were from Jackson Immunoresearch (West Grove, PA). All other reagents were purchased from Sigma (St Louis, MO).

Cell culture and drug treatment
Caco-2 human intestinal cells were obtained from A. Zweibaum (INSERM U.178, Villejuif, France) and grown in DMEM supplemented with 10% FBS, 1% non-essential amino acids, without antibiotics. For experiments, cells were plated on polycarbonate filters with 0.4-μm pores (Transwell no. 3412, Costar Inc., Cambridge, MA) and grown for 2–3 weeks after confluency to permit complete differentiation of the cells (Pinto et al. 1983).

All drugs were prepared in stock solutions at 10 mg/ml in DMSO (dimethyl sulfoxide) and stored frozen at –20 °C. Prior to each experiment, samples were diluted in cold normal medium and drugs used at the following concentrations: colchicine (10 or 20 μM), and nocodazole (10 or 33 μM). After transepithelial resistance (TER) measurement, cells were washed twice with ice-cold PBS containing 1.0 mM CaCl₂ and 1 mM MgCl₂, then incubated in cold normal medium containing the drug for 30 min at 4 °C and warmed up at 37 °C in the presence of the drug for 3 or 6 h. Control experiments were performed in the presence of 0.04% DMSO. In some experiments, filter-grown cells were biotinylated from either the apical or the basolateral side as described (Sargiacomo et al. 1989) and then exposed to the drug. For inhibition of protein synthesis, cycloheximide was used at 50 μg/ml.

Antibodies
Rabbit polyclonal antibodies against human placental alkaline phosphatase were from Accurate (Westbury, NY) and a monoclonal antibody against human transferrin receptor (TFR) was from Boehringer (Mannheim, FRG). Mouse monoclonal antibodies against sucrase (HSI 9), isomaltase (HSI 14), aminopeptidase N (HBB2/45), and dipeptidylpeptidase IV (DAS219) have been described (Beaulieu et al. 1991) and were a generous gift from A. Quaroni. Monoclonal antibody 525-5-4 against Ag 525 and Mab 517 against antigen 517 have been previously characterized (Le Bivic et al. 1987, 1988) and were a gift from A. Le Bivic. Actin filaments were revealed by using FITC-phalloidin at 1 μg/ml.

Indirect immunofluorescence and laser scanning confocal microscopy (LSCM)
Cells grown on filters were fixed, processed and observed by simple indirect immunofluorescence as already described (Gilbert et al. 1991). For double indirect immunofluorescence, combination of FITC-conjugated second antibodies and TR-avidin was used. Epifluorescence examination was performed with a Nikon Optiphot microscope using a x100 lens (1.4 numerical aperture). LSCM was obtained by a Phoibos 1000 unit attached to the microscope (Sarastro, Molecular Dynamics, Sunnyvale, CA). To detect simultaneously FITC- and TR-labeled antigens, samples were excited at 514 nm and emitted light between 525 and 555 nm was recorded for FITC and above 630 nm for TR. The cell monolayer was optically sectioned in horizontal (x–y) or vertical (x–z) planes. Images generated by the Personal Iris graphics workstation (Silicon Graphics, Mountain View, CA) and Sarastro software were photographed from the screen using a Minolta ×500 camera and a 200 mm objective. Kodak Tmax 100 and Ektar 125 films were used.

Electron microscopy
Control and drug-treated cells grown on filters were processed for ultrastructural observation as described (Gilbert et al. 1991). In some experiments, to detect any compartment in communication with the apical or intercellular space, cells were postfixed from the apical or basolateral side of the filter in 2% lanthanum nitrate, 1% OsO₄ in 0.1 M S-collidine buffer at pH 7.2 for 2 h according to Revel and Karnovsky (1967). This staining was also used to enhance the basolateral plasma membrane contrast. Samples were then dehydrated and embedded in Epon. Ultrathin sections were cut perpendicular to the filter and viewed with a JEM electron microscope (JEOL) at 80 kV.

Results

Induction of VACs by microtubule-disrupting drugs
Under normal monolayer culture conditions, the human intestinal Caco-2 cells spontaneously differentiate into columnar enterocyte-like cells with apical brush border and hydrolases (Pinto et al. 1983; Chantret et al. 1988). When confluent filter-grown Caco-2 cells were treated with colchicine at low concentrations (10 μM for 6 h or 20 μM for 3 h), the microtubular network virtually disappeared (Gilbert et al. 1991). A striking feature of colchicine-treated monolayers was the development of large intracellular vacuoles, easily detected by indirect immunofluorescence and LSCM. Vertical optical sections generated by LSCM revealed that these vacuoles were labeled with FITC–phalloidin as strongly as the apical brush border (Fig. 1B). A weak basolateral staining was observed in control and colchicine-treated cells due to the submembranous actin network and seemed to indicate that these vacuoles are intracellular. Basolateral markers were not expressed in these vacuoles (Fig. 1D). These structures were brightly labeled with antibodies against most peptidases known to be expressed on the apical plasma membrane of Caco-2 cells, e.g. sucrase-isomaltase (Fig. 2A, B and D; Table 1). In addition, apical markers were also observed in numerous smaller vesicles (Figs 2C and 3C) located throughout the cytoplasm. Vertical optical sectioning through the vacuoles confirmed their location within the monolayer (Fig. 2B, C and D). To demonstrate the intracellular (as opposed to intercellular) location of these vacuoles, basolateral plasma membranes were localized at the end of the colchicine treatment by biotin labelling and Texas-red-conjugated avidin (Fig. 3). Combination of horizontal (i.e. parallel to the filter) and vertical (i.e. perpendicular to the monolayer) serial sections confirmed their intracellular location and showed no connections with apical or basolateral plasma membranes. Thus, the colchicine-induced large (8–10 μm) intracellular vacuoles are equivalent to the vacuolar apical compartment (VAC) that was previously described in MDCK cells (Vega Salas et al. 1987). VACs are preferentially located at the lower half of the cell (see Figs 2 and 3) but may occasionally occupy the entire cell height (Fig. 1).

| Table 1. Expression of apical and basolateral markers in VACs induced by microtubule-disrupting drugs |
|---------------------------------|---------------------------------|---------------------------------|---------------------------------|---------------------------------|
| Presence in VAC |
| Apical markers: | Actin* | Alkaline phosphatase | Aminopeptidase N | Dipeptidylpeptidase IV | Isomaltase | Mab.517 | Sucrase | Uvomorulin |
| Basolateral markers: | Ag.525 | Transferrin receptor | Uvomorulin |

All the apical markers tested were present in microvillar inclusions. *Actin, also present along the lateral walls, is considered here an apical marker because of the much stronger phalloidin apical staining due to well-developed brush borders.
structures staining with both FITC–phalloidin and apical marker antibodies were observed in ~5% of the cells.

To characterize these VACs further, we examined colchicine-treated cells by electron microscopy. Fig. 4 shows a large VAC (diameter ~6 μm), lined with a complete brush border and a terminal web, in the cytoplasm of a Caco-2 cell. VAC microvilli are 2 μm in length, as are those facing the external medium. The internal lumen is filled with a flocculant slightly electron-dense material and some small vesicle-like structures. Cells containing VACs usually have a very reduced apical surface, with few microvilli still present above the tight-junctional complexes, which results in a pyramidal shape. We also observed ectopic microvilli with well-developed core filaments along the lateral walls of the cells, but their length rarely exceeded 1 μm (Fig. 4, top inset). Furthermore, numerous areas containing glycocalyx-like material were seen along the lateral plasma membranes (bottom inset).

The two different colchicine concentrations we used lead to VAC formation in Caco-2 cells. VAC induction was detected within 3 h when cells were treated with 20 μM of the drug. A 10 μM treatment for the same period of time induced the formation of only basolateral brush border and microvilli; 6 h incubation with 10 μM was necessary to detect VACs. Nocodazole (33 μM, 6 h) also induced the formation of VACs and the expression of apical markers on lateral membranes and in intracellular vesicles (data not shown). Upon examination following a 3 h treatment at 33 μM, we observed few intracellular and basolateral areas labeled with apical markers. With a higher dose (50 μM), we did not see an earlier induction of VAC in Caco-2 cells. Colchicine therefore seemed to be more effective in VAC induction than nocodazole in these cells.

**Origin of VACs**

Experiments were designed to test whether VACs were derived from internalized plasma membranes or were newly synthesized. To examine the first hypothesis we used the following protocol. Filter-grown Caco-2 cells were labelled from the apical or basolateral side with a biotin analog at 4°C (Sargiacomo et al. 1989), incubated in the presence of microtubule-disrupting drug for 6 h to induce VACs, and processed for double indirect immunofluorescence with an apical marker/FITC–second antibody and TR–avidin (Fig. 5). Apical or basolateral biotinylation prior to drug treatment never resulted in the detection of biotin within VACs (Fig. 5B and D) or the numerous smaller intracytoplasmic vesicles expressing apical markers (Fig. 5A and C). We conclude that VACs are not formed by internalization of apical or basolateral plasma membranes. These experiments provided additional evidence that VAC-expressing cells have reduced (Fig. 5D) or practically absent (Fig. 5C) free apical surfaces. However, as proposed by Achler et al. (1989) using an in vivo system, newly synthesized basolateral brush border might become internalized in brush border vacuoles during colchicine treatment. To check this possibility, Caco-2 cells were treated for 3 h with colchicine (10 μM), incubated for another 3 h with the drug in presence of sucrase-isomaltase antibodies added to either the apical or basolateral media. Cells were then processed for double indirect immunofluorescence with Texas-red-conjugated goat anti-mouse and FITC–phalloidin. VACs induced...
between 3 and 6 h do not incorporate antibodies against sucrase-isomaltase, suggesting that neither apical nor basolateral surfaces are internalized to form VAC (data not shown).

To test the involvement of newly synthesized proteins in the formation of VACs, we attempted to induce VACs in the presence of cycloheximide. Cells treated with colchicine and cycloheximide for 6 h did not accumulate apical markers intracellularly or at the basolateral plasma membrane and were not induced to form VACs (data not shown). Thus, VACs are newly synthesized or their formation requires newly synthesized proteins (e.g. for the fusion event; see Discussion). Because no intracellular or basolateral staining with apical marker antibodies was detected upon cycloheximide treatment, we favor de novo formation as the mechanism responsible of VAC formation.

VAC behavior upon drug removal

Filter-grown Caco-2 monolayers where VACs had been previously induced with colchicine or nocodazole were washed twice in drug-free medium and incubated for 24 h in normal medium. Cells were then fixed and processed for electron microscopy or indirect immunofluorescence. For
Fig. 4. Electron micrographs of filter-grown Caco-2 monolayers treated with colchicine for 6 h. In the cell delimited by the broken lines, a large vacuolar apical compartment (VAC) is present and has an infranuclear location. VACs are characterized by the presence of a well-developed brush border facing an internal lumen (*), and are surrounded by a terminal web where microvilli are anchored (arrowheads). This VAC has a diameter of about 6 μm. The apical external surface of the cell exhibiting a VAC almost disappeared (arrow, upper left) but the junctional complexes were still visible. Open arrows indicate the interface between the filter and the basal plasma membrane. Numerous microvilli along the lateral cellular walls (top inset) and glycocalyx-like structures (bottom inset) were found protruding into the intercellular space. Bars: 2 μm; 1 μm, top inset; and 0.5 μm, bottom inset.
both drugs, the microtubular network reappeared and no more VACs were observed after a one-day recovery period. After nocodazole treatment and removal, electron-microscopic examination revealed that the intercellular space was filled with numerous vesicle-like structures and was clearly dilated in some areas (*) but the junctional complexes remained tight (arrowhead). Some structure appeared to be degenerate VACs that had fused with the lateral space (arrow). After a 12 h recovery period, indirect immunofluorescence for sucrase-isomaltase (inset) showed bright apical staining and discontinuous internal labeling, suggesting a fusion of the VAC and the lateral plasma membrane. Bars: 2.5 μm A and B; and 5 μm, each inset.

Discussion

The occurrence of intracellular lumina has been reported in many malignant epithelial cells but is rare in normal cells (for review, see Remy, 1986). An example of this process has been described in vitro in the epithelial cell line HT-29 derived from a human adenocarcinoma (Remy et al., 1984). This system was used to study epithelial differentiation and polarity. Briefly, undifferentiated HT-29 cells (grown in the presence of glucose) form multilayers and develop intracellular and intercellular cysts lined with brush border (Zweibaum et al., 1985; Pinto et al., 1988). Upon replacement of glucose with galactose, HT-29 cells differentiate, lose their VACs, and acquire a polarized phenotype (Remy et al., 1984; Zweibaum et al., 1985; Le Bivic et al., 1988). On the other hand, the human colon carcinoma cell line Caco-2, when grown in glucose-supplemented medium, display a differentiated phenotype and no intracellular lumina (VACs) or intercellular cysts (Pinto et al., 1988; Chantret et al., 1988). In vivo, transient secondary intercellular lumina were also described during differentiation of the fetal rat colon epithelium, but no intracellular lumina were observed (Colony and Neutra, 1983).

No clues exist on the biogenesis of these large microvillar inclusions. The results of this report using Caco-2 cells suggest that VAC is a newly synthesized compartment, rather than a compartment derived from the pre-existing brush border. A similar origin was proposed (with no experimental evidence) for the brush border vesicles observed in Davidson's disease (Cutz et al., 1989).

In vivo treatment of rats with colchicine has also been shown to induce the formation of brush border vacuoles in intestinal cells (Achler et al., 1989). The proposed mechanism in this case was the internalization of newly synthesized brush borders misplacated to the basolateral surface due to microtubular disruption and consequent mis-sorting of apical proteins. In our in vitro system, we clearly demonstrated, using prebiotinylated monolayers, that internalization of neither apical nor basolateral membranes was responsible for VAC formation. Furthermore, antibodies against an apical marker (sucrase-isomaltase) added continuously to the apical or basolateral medium at the time of VAC production did not result in labelling of VACs by the antibody. Thus, in cultured Caco-2 cells, apical components appear to be incorporated into VACs without prior surface appearance. Another difference between native intestinal cells and Caco-2 cells is that, in vivo, VACs fused with the apical surface whereas, in vitro, VACs appear to be degraded. Perhaps, these differences may be attributed to the different experimental protocols utilized in vivo and in vitro (drug was administered to animals by stomach gavage, by the procedure used by Achler et al. (1989) and, therefore, the levels were constantly changing during the time of the experiment) or, alternatively, to differences in behavior in the response of native and cultured cells. Experiments performed on human tissues indicated that not only microtubular disruption but also microfilament disassembly induced similar microvillar inclusions (Carruthers et al., 1986). In this case, collapse of the straight brush border into folds is believed to be the initial step leading to the formation of brush border vacuoles within the cytoplasm (Carruthers et al., 1986). Preliminary results in Caco-2 cells indicated that cytochalasin D treatment can generate small VAC-like structures (data not shown). Their location close to the apical surface seemed to support this hypothesis.

Recent studies of the biogenetic pathways of plasma membranes proteins in Caco-2 cells have shown that about 10 % of several newly synthesized apical proteins failed to reach the cell surface during colchicine treatment (Gilbert et al., 1991). The results of this morphological study suggest that these proteins are delivered to VACs or intracellular vesicles rather than to the apical surface. This is consistent with the de novo origin of the VAC we proposed. VAC formation may involve fusion between post-Golgi vesicles. It is interesting to note that Caco-2 VACs expressed all apical markers previously detected in this cell line (Matter et al., 1990; Le Bivic et al., 1990; Gilbert et al., 1991), independently of whether these markers follow direct (e.g., sucrase-isomaltase) or indirect (e.g., dipeptidylpeptidase IV) pathways to the cell surface. Only 5 % of colchicine-treated Caco-2 cells expressed VACs. The reason for this low percentage of cellular response to colchicine is unclear. One possibility is that...
the ability to form VACs is expressed permanently by only a small percentage of cells, in spite of the uniform disruption of the microtubular network caused by colchicine. If this is the case, it should be possible to clone ‘VAC-forming cells”; we are currently attempting to do this. Alternatively, the VAC-forming ability may be a stochastic phenomenon for a given cell population. The availability of a model system in which brush-border-containing vesicles may be developed in controlled in vitro experiments opens the way towards an understanding of the biogenesis of the apical domain in normal cells and in pathological states, such as Davidson’s disease.

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