Formation of Multilayers in the Caco-2 Cell Culture Model: A Confocal Laser Scanning Microscopy Study

Barbara Rothen-Rutishauser,[1,2] Annette Braun,[1] Maja Günthert,[1] and Heidi Wunderli-Allenspach[1,2]

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Purpose. To introduce confocal laser scanning microscopy (CLSM) combined with digital image restoration to characterise Caco-2 cells under different culture conditions, and thus to define additional valid criteria for the optimisation of culture models.

Methods. Growth curves were established and transepithelial electrical resistance (TEER) measured for cells grown in EMEM or DMEM medium on Cyclopore® membranes. Cytoskeleton, cell nuclei and tight junctions (TJ) were investigated by CLSM.

Results. Cultures reached a plateau of $\sim 4.5 \times 10^5$ cells/cm^2 after $\sim$ 10 days. At the same time TEER reached 750 Ω cm^2. An irregular, fairly complete network of TJ was present at confluence ($\sim$2 d). Between 15 and 30 days a regular TJ network was established. Cells formed mixed mono- and multilayers under most conditions with two exceptions: flat monolayers were observed on polycarbonate filters with EMEM and with the Biocoat® intestinal epithelium differentiation environment system. In multilayers TJ were found in the upper as well as in the lower cell layers although the regular vertical polarity was disturbed.

Conclusions. CLSM represents an important tool to investigate the cytoarchitecture of Caco-2 cells. 3D-analysis of confocal data gives important clues on the characteristics of cell layers and thus helps to validate optimisation strategies.

KEY WORDS: Caco-2; in vitro intestinal epithelial model; confocal laser scanning microscopy; tight junctions; cytoarchitecture; variability.

INTRODUCTION

The human colon adenocarcinoma cell line Caco-2 has gained enormous popularity as an in vitro tool for the study of drug absorption and metabolism in the intestinal mucosa (1,2). Results of fluxes of a range of passively absorbed compounds in Caco-2 cells correlate well with those obtained from human colonic segments mounted in diffusion chambers (3,4). The apparent permeability (P_{app}) values calculated from transport studies with Caco-2 cells show good correlation with human intestinal absorption (5).

A major disadvantage of the Caco-2 model is the large interclonal variability since the cells have the tendency to alter their phenotype upon subculture (1). Additionally the differences of expression levels and substrate specificities of the carriers and efflux systems found in Caco-2 cells in comparison to the in vivo situation have to be considered (6).

The current intestinal cell culture models are far from perfect and refinements are needed to better predict human intestinal permeability. In the process of improving existing and designing new intestinal models the traditional cell biology is estimated to play an important role also in the future (7). This includes optimisation of microenvironmental factors such as the extracellular matrix and growth medium as well as the definition of age-related stages of cells for experimental cultures (8).

To follow the optimisation of cultures quantitatively, valid criteria have to be established such as the cytoarchitecture of cells and the formation of tight junctions (TJ). Immunofluorescence methods combined with confocal laser scanning microscopy (CLSM) represent an important tool for the characterisation of cell cultures (8). It permits to look at the 3D structure of cell layers and to localise specific molecules like carriers or structural proteins. In this paper we used this method to characterise Caco-2 cells under different, widely used culture conditions. By applying a deconvolution algorithm to data sets recorded at high magnification, cell layers could unambiguously be identified as either mono- or multilayers. In addition, the completeness of the TJ network was tested. The relevance of these findings is discussed in view of transport studies.

MATERIALS AND METHODS

Cell Cultures

Caco-2 cells (passage 16) were purchased from the American Type Culture Collection (ATCC, Rockville, MD). All experiments were performed between passage 28 and 60. If not stated otherwise, cells were grown at 37°C in a 5% CO_2 atmosphere according to the ATCC recommendations, i.e. in Minimum Essential Medium with Earle's Salts (EMEM), non essential amino acids and L-glutamine (Gibco BRL # 41500-018), supplemented with sodium bicarbonate (Fluka # 71628), sodium pyruvate (Fluka # 15990), penicillin-streptomycin (Gibco BRL # 15140-114) and 20% foetal calf serum (PAA Laboratories GmbH, Linz, Austria # A 15-042). They were propagated on plastic Falcon®-flasks (Winiger AG, Fisher Scientific Company, Wohlen, Switzerland) and subcultivated twice weekly. Unless otherwise stated experimental cultures were seeded at 10^5 cells/cm^2 on Falcon® Cell Culture Inserts with a Cyclopore® membrane (4.19 cm^2, 0.4 μm pores, #3090; Becton Dickinson) in Falcon® 6-well plates (Winiger AG) with 2.5 ml medium in the upper, and 3 ml in the lower chamber. To establish growth curves cells were propagated in TPP® 6-well plates (Winiger AG). Cells were trypsinized and cell numbers counted in a Neubauer chamber. The transepithelial electrical resistance (TEER) was determined at 37°C using a Millicell-ERS system (MERS 000 01, Millipore) as previously described (8). In some cases, as indicated, cells were grown in Dulbeccos MEM (DMEM) with non essential amino acids (Gibco BRL # 10938-025), L-glutamine (Gibco BRL # 25030-024), sodium pyruvate, penicillin-streptomycin, sodium bicarbonate and 10% foetal

[1] Biopharmacy, Department of Applied Biosciences, ETH Zurich, CH-8057 Zurich, Switzerland.

[2] To whom correspondence should be addressed. (e-mail: wunderli@pharma.ethz.ch)

ABBREVIATIONS: CLSM, confocal laser scanning microscopy; TJ, tightjunction(s); ZO, zonula occludens; TEER, transepithelial electrical resistance.

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calf serum. Beside the Cyclopore® membranes (see above), Snapwell™ Inserts (polycarbonate, 1 cm^2, 0.4 μm pores, # 3407; Costar) were used alternatively as indicated.

For comparison the Biocoat® intestinal epithelium differentiation environment (Becton Dickinson, # 30057M) was studied (9). In this case, Caco-2 cells were seeded onto a fibrillar collagen coated insert (4.19 cm^2, 1 μm pores) at high density, i.e. 6.5×10^5 cells/cm^2, and grown in serum free DMEM with MITO+™ serum extender. Starting at 24 h post-seeding the medium was replaced daily with Entero-STIM™ (butyric acid containing serum free DMEM). MDCK cells were grown as described in Rothen-Rutishauser et al. (8). Briefly, cells were seeded at a concentration of 5×10^4 cells/cm^2 and experimental cultures were also propagated on Falcon® Cell Culture Inserts with a Cyclopore® membrane. They were grown in Eagle's minimum essential medium (MEM) with Earl's salts supplemented with 20 mM HEPES (ICN Biomedicals Inc.) and 10% foetal calf serum for 11 days, which corresponds to stage III cells.

Immunofluorescent Labeling

For immunofluorescence studies cells were labelled and prepared as previously described (8). Briefly, cell layers were fixed for 15 min at room temperature in 3% paraformaldehyde in PBS (10 mM phosphate buffered saline pH 7.4: 130 mM NaCl, Na_2HPO_4, KH_2PO_4), treated with 0.1 M glycine in PBS for 5 min and permeabilised in 0.2% Triton X-100 in PBS for 15 min. Samples were incubated at 37°C with the first antibody for 60 min and with the second antibody for 90 min. Preparations were mounted in 0.1 M Tris-HCl (pH 9.5) : glycerol (3:7) containing 50 mg/ml n-propyl-gallate (Sigma, # 3130).

Antibodies were diluted in PBS containing 3% bovine serum albumin as follows: anti ZO1 1:100 (Zymed Laboratories), anti α-tubulin (Clone DM 1A) 1:100 (Sigma), anti rat cyanine 5 and anti mouse cyanine 5 both 1:50 (Chemicon). The dilution was 1:100 both for TRITC-phalloidin (Molecular Probes) and for the DAPI stain (4,6-Diamidino-2-Phenylindole; Hoechst, #333342).

Confocal Laser Scanning Microscopy

Confocal data sets were sampled on a Zeiss LSM 410 inverted microscope. Image processing was done on a Silicon Graphics workstation with the IMARIS software (Bitplane AG, Zurich, Switzerland, *http://www.bitplane.ch*). In an effort to improve the resolution of images recorded at high magnification and to reduce noise, data restoration was done with the Huygens 2 software (Scientific Volume Imaging B. V., Hilversum, Netherlands, *http://www.svi.nl*). A theoretical Point Spread Function (PSF) was computed using a PSF generator image as described in van der Voort and Strasters (10).

RESULTS

Growth Characteristics of Caco-2 Cells

Cells were cultured on Cyclopore® membranes with EMEM. They grew confluent within 2 to 3 days. After 10 days the cell number reached a plateau of 4 to 4.5×10^5 cells/cm^2 and stayed in this range till day 30 (Fig. 1a). At later times cells detached from the substrate and the number of attached cells decreased (Fig. 1a). TEER values increased continuously and reached a plateau between 750 and 800 Ω cm^2 after 10 days (Fig. 1b). Values decreased as expected from the growth curve after 30 days. If cells were grown in DMEM on Cyclopore® membranes, no significant differences were found for the growth curve and TEER values, respectively (data not shown). The following experiments were performed with cultures grown on Cyclopore® membranes with EMEM unless otherwise stated.

Tight Junction and Cytoskeletal Organisation

Caco-2 cells were prepared for CLSM at various time points and stained with an antibody against the TJ protein zonula occludens 1 (ZO1). In sub-confluent cultures, i.e. after 1 day, ZO1 was found along the cell borders where cell-cell contacts had been established (Fig. 2a). After 4 days a confluent cell layer was formed with an irregular, but fairly complete TJ network close to the apical cell surface (Fig. 2b). The same pattern was also observed after 11 days in culture when the stationary phase was reached (Fig. 2c). After 28 days a regular network of ZO1 was detected between neighbouring cells (Fig. 2d). The staining for occludin, another TJ protein, revealed the same development of the TJ pattern (data not shown). The F-actin cytoskeleton was visualised with TRITC-phalloidin. In fully differentiated, polarised cells, i.e. 28 days after seeding (Fig. 2e), F-actin staining was mostly concentrated near the apical side of the cells, where the typical brush border microvilli, each containing bundles of microfilaments, are localised. Two cell types already described by Pinto et al. (11) could be observed in the confocal microscope: cells with a thick carpet of villi (Fig. 2e, arrow), and cells with flower-like clusters of villi with apical ends sticking together (Fig. 2e, asterisk).

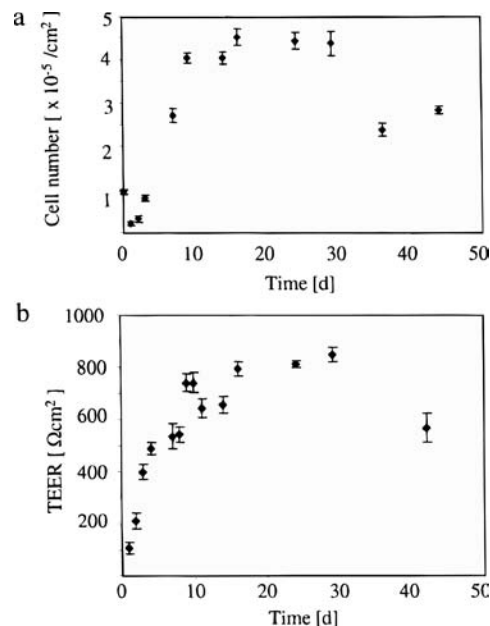


Fig. 1. Growth characteristics of Caco-2 cells in culture. Cells were grown in EMEM on Cyclopore® membranes. Cell numbers (a) and TEER (b) were determined at various times after seeding. Each point represents the mean ± standard deviation of 5 individual culture wells.

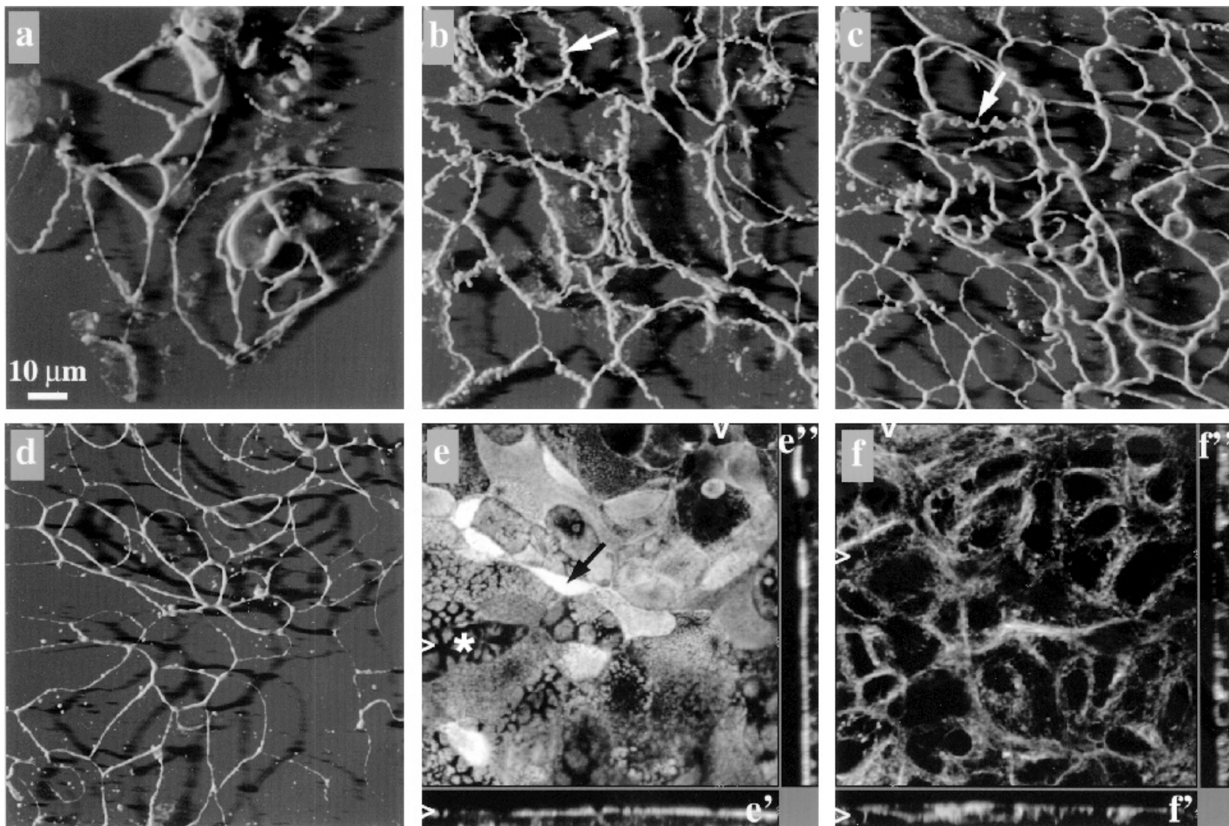


Fig. 2. Tight junction formation and cytoskeletal organisation. Caco-2 cells were cultured in EMEM on Cyclopore® membranes for 1 day (a), 4 days (b), 11 days (c), and 28 days (d, e, f) and stained for ZO1 (a, b, c, d), F-actin (e), and α-tubulin (f). The arrows in (b) and (c) point to TJ that form irregular lines. The arrow in (e) points to a cell with a thick carpet of villi, the asterisk in (e) marks a cell with flower-like clusters of villi. (a, b, c, d) 3D reconstructions; (e, f) xy- projections; (e′, f′) xz-projections; (e″, f″) yz-projections. Open arrows in (e) and (f) mark the position of projections.

Microtubules were concentrated in the perinuclear area as well as throughout the cytoplasm as a thin network (Fig. 2f).

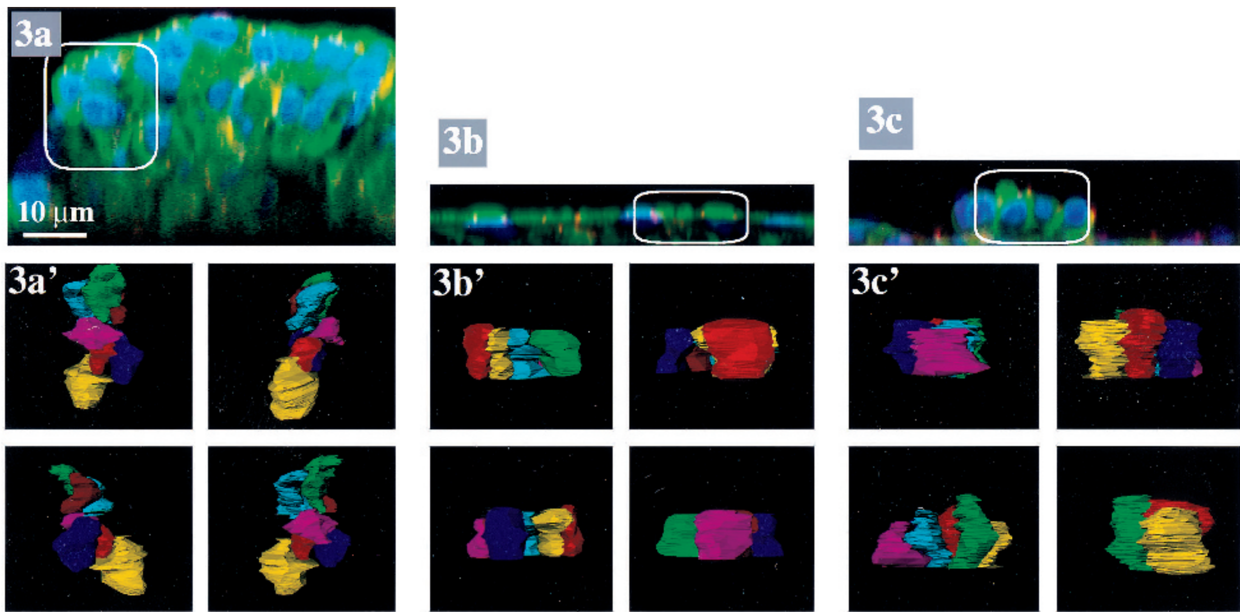
Development of Multilayers in Caco-2 Cell Cultures

To distinguish between the formation of mono- and multilayers respectively, Caco-2 cells were cultured and prepared for CLSM as indicated. Phase contrast microscopy showed that no cell clusters were present at seeding. With the Depth Analyse program of the IMARIS software, representative confocal data sets were chosen and the contours of a few neighbouring cells determined. The resulting polygons were colour-coded and visualised at different angles. With this approach the formation of multilayers in Caco-2 cell cultures grown in EMEM on Cyclopore® membranes could be demonstrated unambiguously (Fig. 3a, a′). About half of the area of the culture insert was covered by multilayers, the other one by monolayers (Fig. 3b, b′). For comparison we analysed Madin Darby Canine Kidney (MDCK) cells at stage IV (8) by the same approach (Fig. 3c, c′). In this case, the typical uplifted monolayers, i.e. the dome formation, was seen.

One confocal data set was recorded at high resolution and a deconvolution algorithm was applied to reduce noise and to

Fig. 3. (*opposite*) Formation of multilayers in Caco-2 cells. Caco-2 cells were cultured in EMEM on Cyclopore® membranes for 28 days (a, b, d, e, f), MDCK cells for 28 days (see Methods) (c) and triple-stained for F-actin (green), ZO1 (red), and the cell nuclei (blue). (a, b, c) optical sections trough the z-axis. Contours of few neighbouring cells (marked area in a, b, c) were chosen, the resulting polygons were colour-coded and visualised at different angles (a′, b′, c′). (d) and (e) optical sections through an identical data set after deconvolution at different depth of the layer (black arrowhead in d, e), whereas the insets show only ZO1 (red). (d, e) xy-projections; (d′, e′) xz-projections; (d″, e″) yz-projections. A 3D reconstruction of ZO1 (red) and the cell nuclei (blue) of the same data set is shown top-down in (f), and bottom-up in (f′). Open arrows in (d) and (e) mark the position of projections.

Fig. 4. (*opposite*) Influence of culture conditions on the formation of multilayers. Caco-2 cells were grown for 28 days (a, b) or 3 days (c), and triple-stained for F-actin (green), ZO1 (red), and the cell nuclei (blue). (a) DMEM on Cyclopore® membranes; (b) EMEM on polycarbonate filters; (c) Biocoat® intestinal epithelium differentiation environment. (a, b, c) xy-projections; (a′, b′, c′) xz-projections; (a″, b″, c″) yz-projections. Open arrows in (a), (b) and (c) mark the position of projections.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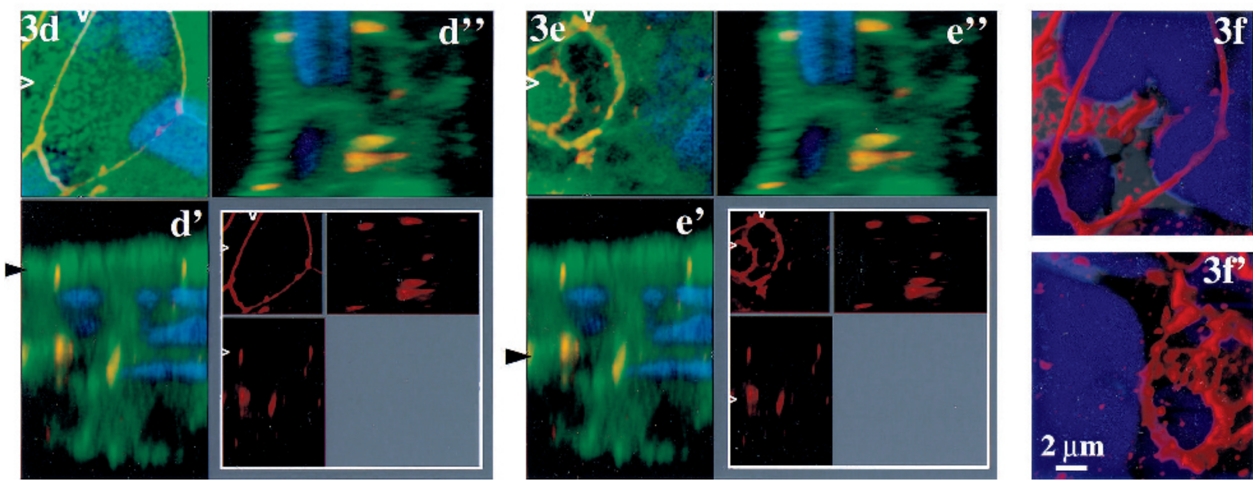
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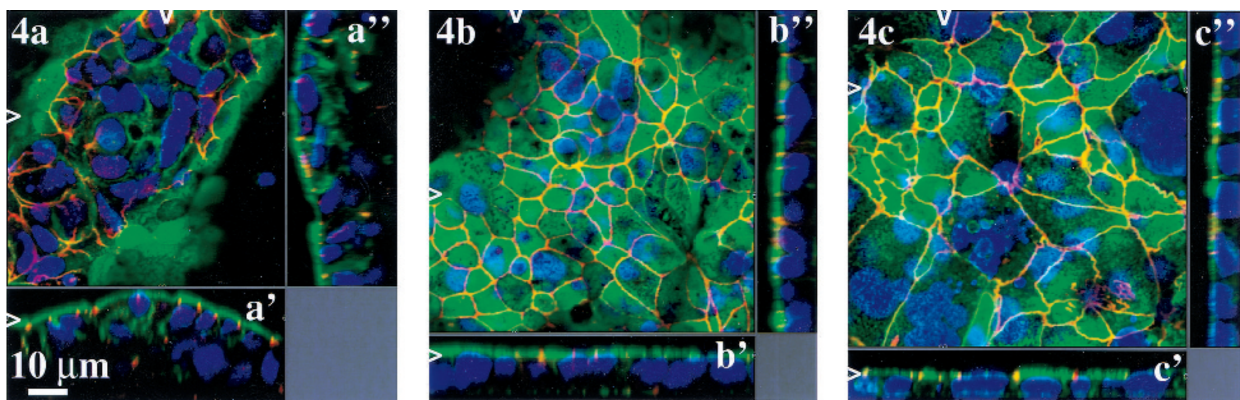
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increase the resolution of the images, especially in the z-axis. (Fig. 3d, e, f). The cells were triple-stained for F-actin (green), the TJ protein ZO1 (red), and the cell nuclei (blue). Fig. 3d and 3e show optical sections through an identical data set at different depth of the layer. TJ could be seen near the apical side in cells that are most distant from the substrate (Fig. 3d) as well as in the middle part of the cell layer (Fig. 3e). A 3D reconstruction of the same data set is shown top-down in figure 3f, and bottom-up in figure 3f′ to clearly show TJ in the lower cell layer.

Various established culture conditions were tested to study their influence on the formation of multilayers. No significant difference was found between cells grown in DMEM instead of EMEM on Cyclopore® membranes (Fig. 4a) or on Cyclopore® membranes coated with collagen I (data not shown). A homogeneous monolayer was, however, reproducibly formed when the cells were cultured with EMEM on polycarbonate filters (Fig. 4b). For comparison the Biocoat® intestinal epithelium differentiation environment was also studied. At 3 days after seeding a regular monolayer was found (Fig. 4c).

DISCUSSION

CLSM in combination with data restoration has been successfully applied to distinguish between mono- and multilayer formation in Caco-2 cell cultures under various culture conditions. The 3D analysis of confocal data sets, in particular the reconstruction of the z-axis gives important clues on the characteristics of a cell layer. Although phase contrast microscopy in general is an important tool in cell culture, it is of limited use as soon as confluence is reached and thicker cell layers have to be investigated. In the past, electron microscopy has often been applied to study cross sections of cell layers (12,13). However, this approach is very time consuming since laborious methods have to be applied if specific labelling is needed. CLSM in conjunction with immunofluorescence methods provides an important tool in the effort to optimise cell culture models since several fluorescent markers can be used simultaneously.

The high variability in the phenotype of Caco-2 cells (1) could be confirmed not only for different growth conditions, but also within one culture where patches of mono- and multilayers were found in close vicinity. In multilayers TJ are not restricted to the most apical cell layer, but also appear in lower layers although not as a horizontal network. This means that the polarity of cells (11) is less pronounced or even lost. The consequences of this phenomenon for the transport of drugs have to be studied carefully. Although the effect is minimal in the case of passive permeation (unpublished results), carrier-mediated processes could be influenced significantly.

When cells were seeded on a polycarbonate filter instead of the Cyclopore® membrane they formed monolayers with both EMEM and DMEM medium. However, establishment of fully differentiated cells with a regular network required a culture period of three weeks. CLSM studies of cells cultured with the Biocoat® system consisting of collagen inserts and a special serum free medium revealed that this optimised intestinal epithelium differentiation environment (9) also grew in homogeneous monolayers. Already within 3 days a regular TJ network was established. It is interesting to note that the same effect is not reached under the same condition if the Biocoat medium is changed to EMEM (data not shown). Special defined media, i.e.serum free DMEM with MITO+™ serum extender and Entero-STIM™, are needed for the optimal effect. It remains open to what extent the different transporters are expressed in the Biocoat® system within 3 days. These results clearly demonstrate the influence of the type of culture membrane and the medium on the differentiation and cytoarchitecture in Caco-2 cells. If monolayers are needed, Caco-2 cells are best grown on polycarbonate filters with either EMEM or DMEM medium, or alternatively, the Biocoat® system is used.

It has been reported that growth rate, TEER and sucrase-isomaltase activity increase as passage number increases (13). Based on this study it has been proposed that the optimum passage range for experimental purposes is 28 to 65. In agreement with this report we did not find any significant difference in the growth curve and TEER measurement of cells between passage 28 and 60. The formation of multilayers was also not passage number-related. The mixed mono- and mulitlayer phenotype was found in early and late passages. However, significant passage number-related differences have been reported in the expression of transporters such as P-glycoprotein (14). This is certainly relevant for transport studies with P-glycoprotein substrates and inhibitors.

The cytoarchitecture as revealed by labelling of F-actin and nuclei is a helpful criterion for comparison of various phenotypes. Another important aspect is the formation of TJ. The expression of TJ proteins can be tested, e.g. by Western blot analysis, however, to act as a barrier the TJ proteins not only have to be produced, but also have to be correctly inserted at the cell-cell contacts, which can easily be checked by CLSM. This approach ideally complements studies on paracellular transport with test molecules, e.g. mannitol, and TEER measurements that are used to test the tightness of cell layers.

As small changes in the growth conditions can lead to significant differences in the phenotype of Caco-2 cells and other cell cultures, it is mandatory to develop standard protocols regarding growth of cells for transport studies. A complete characterisation of cell cultures with respect to TEER, growth curve and mannitol transport, complemented by CLSM studies for the cytoarchitecture and localisation of the TJ at different age-related stages helps to validate different culture models. The sophisticated software available for data analysis and reconstruction provides a whole range of parameters for quantitative comparisons.

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