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PHENOTYPIC CHARACTERIZATION OF HUMAN UMBILICAL VEIN ENDOTHELIAL (ECV304) AND URINARY CARCINOMA (T24) CELLS: ENDOTHELIAL VERSUS EPITHELIAL FEATURES

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SUMMARY

ECV304 cells reported as originating from human umbilical vein endothelial cells by spontaneous transformation have been used as a model cell line for endothelia over the last decade. Recently, deoxyribonucleic acid fingerprinting revealed an identical genotype for ECV304 and T24 cells (urinary bladder carcinoma cell line). In order to resolve the apparent discrepancy between the identical genotype and the fact that ECV304 cells phenotypically show important endothelial characteristics, a comparative study was performed. Immortalized porcine brain microvascular endothelial cells/C1–2, and Madin Darby canine kidney cells were included as typical endothelial and epithelial cells, respectively. Various methods, such as confocal laser scanning microscopy, Western blot, and protein activity tests, were used to study the cell lines. ECV304 and T24 cells differ in criteria, such as growth behavior, cytoarchitecture, tight junction arrangement, transmembrane electrical resistance, and activity of γ-glutamyltransferase. Several endothelial markers (von Willebrand factor, uptake of low-density lipoprotein, vimentin) could clearly be identified in ECV304, but not in T24 cells. Desmoglein and cytokeratin, both known as epithelial markers, were found in ECV304 as well as in T24 cells. However, differences were found for the two cell lines with respect to the type of cytokeratin: in ECV304 cells mainly cytokeratin 18 (45 kDa) is found, whereas in T24 cells cytokeratin 8 (52 kDa) is predominant. As we could demonstrate, the ECV304 cell line exposes many endothelial features which, in view of the scarcity of suitable endothelial cell lines, still make it an attractive in vitro model for endothelia.

Key words: endothelia; epithelia; tight junction; cytoarchitecture; cell markers.

INTRODUCTION

Endothelial cells are involved in a wide range of pathological processes including inflammation (Abbot et al., 1992), tumor invasion (Folkman, 1992), and atherosclerosis (Massy and Keane, 1996). This has led to considerable efforts to isolate and culture endothelial cells from both human and animal sources in order to further investigate their role. One major problem of primary cell cultures is their heterogeneity and the loss of specific markers during cultivation (Scott and Bicknell, 1993; De Boer et al., 1999) which makes comparison of the results between different studies difficult. For reproducibility and easy handling, stable cell lines are a valid alternative under the assumption that standard culture conditions are established, and the resulting phenotype is characterized with regard to specific markers. Only a few endothelial cell lines, particularly of human origin, are available today. This is the reason why ECV304 cells, a spontaneously transformed human umbilical vein endothelial cell line (Takahashi et al., 1990), became very popular for studies on angiogenesis (Hughes, 1996), cell migration (Kikkawa et al., 1996), glucose transport studies (Vinals et al., 1999), or signal transduction of the vascular growth factor (Abedi and Zachary, 1997). In addition, the ECV304 cells were often used for investigation on the blood–brain barrier (BBB) as they express many characteristic BBB features, especially under the influence of glial cells (Hurst and Fritz, 1996; Dobbie et al., 1999).

The origin of ECV304 cells has recently been questioned by the finding that the deoxyribonucleic acid (DNA) fingerprints of ECV304 are identical with those of the T24 cell line (Dirks et al., 1999). As T24, a urinary bladder cancer cell line, was established before the ECV304 cell line (Bubenik et al., 1973), ECV304 has to be regarded as a T24 variant clone that arose by cross-contamination of the endothelial culture. DNA fingerprint screening was performed independently at the DSMZ (German Collection of Microorganisms and Cell Cultures), ATCC (American Type Culture Collection), ECACC (European Collection of Animal Cell Cultures), and JCRB (Japanese Collection of Research Bioresources) with the same outcome: i.e., an identical banding pattern of the two cell lines.

Although an identical genotype has been established for ECV304 and T24 cells, it is clear from the published data that ECV304 cells express some endothelial characteristics (Takahashi et al., 1990; Takahashi and Sawasaki, 1991; Lechardeur et al., 1995; Hughes, 1996; Dobbie et al., 1999; Kiessling et al., 1999). Recognizing the importance of ECV304 for endothelial studies in the present situation, a systematic investigation on the phenotype of the two cells

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was undertaken. For comparison, Madin Darby canine kidney (MDCK) cells (McRoberts et al., 1981), a typical epithelial cell line, and porcine brain microvascular endothelial cells (PBMEC)/C1–2 cells (Teifel and Friedl, 1996), which are of endothelial origin, were included. ECV304 and T24 cells were first investigated with regard to general features, such as growth behavior, cytoarchitecture, expression of P-glycoprotein (P-gp) and γ-glutamyltransferase (γ-GT activity). The arrangement of tight junctions (TJ) (Bowman et al., 1992; Dejana et al., 1995; Dunina-Barkovskaya, 1998) was explored by means of confocal laser scanning microscopy (CLSM) and transmembrane electrical resistance (TEER) measurements. The cells were then investigated with respect to specific endothelial characteristics. As a typical endothelial feature, the uptake of acetylated low-density lipoprotein (LDL) was measured as well as the expression of von Willebrand factor (vWF), platelet endothelial cell adhesion molecule-1 (PECAM-1) (Hewett and Murray, 1993), VE-cadherin, and vimentin. For comparison, some epithelial markers were also studied, such as E-cadherin, cytokeratin, and desmoglein.

Materials and Methods

Cell culture. The following cells were used: ECV304 (ATCC CRL-1998; passage #133–150), T24 (ATCC HTB-4; passage #42–50), MDCK (Rothen-Rutishauser et al., 1998; passage #216–270), and PBMEC/C1–2 (kindly provided by Prof. Friedl; Teifel and Friedl, 1996; passage #91–198). No difference was found in any cell line between the early and late passages. Cell monolayers were propagated on plastic TPP® flasks (Winiger AG, Wohlen, Switzerland). The media were M199 (Sigma Chemical Co., Buchs, Switzerland) with 10 m*M* *N*-2-hydroxyethylpiperazine-*N*′-2-ethane-sulfonic acid (HEPES) (Sigma) for ECV304, PBMEC/C1–2, and T24 cells, and Eagle's minimum essential medium with Earl's salts supplemented with 2 m*M* L-glutamine (GIBCO BRL Life Technologies, Basel, Switzerland) and 0.225% $NaCO_3$ for the MDCK cells. In addition to M199, McCoy's 5A medium (Sigma) with 2 m*M* L-glutamine (GIBCO BRL) was used to test the differences in growth and electrical resistance in T24 cells. All media contained 10% fetal calf serum (PAA Laboratories GmbH, Linz, Austria), 100 units penicillin/ml, and 100 μg streptomycin/ml (Penicillin–Streptomycin, GIBCO BRL #15140-114). Incubations were in a 5% CO_2 atmosphere at 37° C. In the case of the PBMEC/C1–2 cells, TPP® flasks were coated with 1% porcine skin gelatine (Sigma). In order to establish the growth curves, cells were propagated in TPP® 24-well plates. Cells were trypsinized and counted with a Neubauer counting chamber. Experimental cultures were grown on Falcon® cell culture inserts with a PET® membrane (0.4 μm, 4.3 cm^2, Becton Dickinson, Basel, Switzerland, #3090) with 2.5 ml medium above the insert and 3.0 ml below the insert. Confluence was reached at the same time as with cultures grown on TPP® plastic. For experimental cultures, the medium was changed twice weekly. Electrical resistance was measured with the Millicell-ERS system (MERS 000 01, Millipore, Volketswil, Switzerland) at 37° C about 6 h after medium change.

Confocal laser scanning microscopy. A Zeiss LSM 410 inverted microscope was used with the following lasers: HeNe 633 nm, HeNe 543 nm, Ar 488/514 nm, and Ar UV 364 nm. Optical sections at intervals of 0.3 μm were taken with a ×63/1.4 Plan-Apochromat objective. Image processing was done on a Silicon Graphics workstation using IMARIS, a three-dimensional (3D) multichannel image processing software for confocal microscopic images (Bitplane AG, Zurich, Switzerland).

Antibodies and fluorescent reagents. For CLSM, the following antibodies were used. An anti–ZO-1 polyclonal antibody (Zymed, South San Francisco, USA), an anti–E-cadherin monoclonal antibody (Sigma), and affinity purified secondary antibodies (IgG) conjugated with cyanine 5 (Chemicon, Temecula, CA, USA) or cyanine 3 (Chemicon) were used. F-actin was labeled with phalloidin Oregon Green (Molecular Probes, Eugene, OR, USA). Cell nuclei were visualized with 4,6-diamidino-2-phenylindole (DAPI, Hoechst AG, Frankfurt, Germany). Uptake studies were performed with 1,1′dioctadecyl-3,3,3′,3′-tetramethyl-indocarbocyanine perchlorate acetylated LDL (DiI-Ac-LDL) (Molecular Probes). Antibodies used in Western blots were as follows: anti–P-gp monoclonal antibody (C219) and anti-human vWF polyclonal antibody (DAKO), and antivimentin monoclonal antibody, anti–E-cadherin monoclonal antibody, and anti–Pan-cytokeratin monoclonal antibody (Sigma). The antidesmoglein½ monoclonal antibody was from Progen (PROGEN Biotechnik GmbH, Heidelberg, Germany). For VE-cadherin, an anti–VE-cadherin monoclonal antibody (11D4.1; BD PharMingen, Basel, Switzerland) or, alternatively, an anti–VE-cadherin monoclonal antibody (Chemicon) was used. PECAM-1 was stained with an anti-human monoclonal antibody (WM-59, Sigma) or a monoclonal anti-mouse antibody (MEC 13.3, BD PharMingen). For detection on Western blots, primary antibodies were reacted with the secondary goat anti-mouse IgG, goat anti-rabbit IgG, or goat anti-rat IgG, all coupled to alkaline phosphatase (Pierce, Rockford, USA).

Immunofluorescent labeling. Inserts with cell layers were prepared for CLSM as described (Rothen-Rutishauser et al., 1998). Briefly, cell layers were fixed for 15 min at room temperature in 3% paraformaldehyde (PFA) in phosphate-buffered saline (PBS) pH 7.4 (10 m*M* Na_2HPO_4/KH_2PO_4, 130 m*M* NaCl). They were treated with 0.1 *M* glycine in PBS for 5 min and permeabilized in 0.2% Triton X-100 in PBS for 15 min. Samples were incubated at 37° C for 60 min with the primary antibody, and for 90 min with the secondary antibody. Antibodies were diluted in PBS containing 3% bovine serum albumin as follows: anti–ZO-1, 1:100; anti–E-cadherin, 1:100; anti-rabbit cyanine 5 and anti-rat cyanine 3, each 1:50. The dilution for phalloidin Oregon Green was 1:10, and the concentration for DAPI was 1 μg/ml. Preparations were mounted in 0.1 *M* Tris-HCl (pH 9.5)/glycerol (3:7) containing 50 mg *n*-propyl-gallate per milliliter (Sigma).

DiI-Ac-LDL uptake. The cellular uptake of DiI-Ac-LDL was assessed by the method of Voyta et al. (1984). In brief, cells were incubated with DiI-Ac-LDL (10 μg/ml) in growth medium for 6 h at 37° C, then washed in PBS pH 7.4, and fixed in 3% PFA for 15 min. After washing with PBS, cells were labeled with DAPI for 90 min, and mounted as described above.

Immunoblots. For vWF detection, cells were extracted in gel electrophoresis sample buffer (50 m*M* HEPES, 150 m*M* NaCl, 10% glycerol, 1% Triton X-100, 1.5 m*M* $MgCl_2$-$6H_2O$, 1 m*M* ethylene glycol-bis(aminoethylether)-tetraacetic acid [EGTA], 1 m*M* phenylmethylsulfonyl fluoride [PMSF], 1% aprotinin, and 100 μ*M* benzamidine). Samples were denatured by heating for 5 min at 95° C. For the detection of all other antibodies, crude membranes were prepared as described (Hrycyna et al., 1998). They were not denatured before loading on the gel. Briefly, cells were harvested by scraping in ice-cold PBS containing 1% aprotinin (Sigma). After centrifugation for 5 min at 13,000 × *g* and 4° C, the cells were resuspended in hypotonic lysis buffer (10 m*M* Tris pH 7.5, 10 m*M* NaCl, 1 m*M* $MgCl_2$, and 1% aprotinin) and frozen at −80° C. Subsequently, the cells were thawed and incubated on ice for 30 min. They were then disrupted using 50 strokes with a POTTER®S homogenizer (B.Braun Biotech International, Melsungen, Germany). After differential centrifugation, the membranes were resuspended in buffer containing 10% glycerol and stored at −80° C until use. The protein concentration of each sample was measured with the BioRad assay (BioRad, Glattbrugg, Switzerland). Sodium dodecyl sulfate-polyacrylamide-gel electrophoresis was carried out on either 7.5 or 12% mini-gels (BioRad) with around 30 μg protein per slot. Gels were blotted on to hybond-C nitrocellulose sheets (Amersham, Dübendorf, Switzerland), and the primary antibodies were reacted with the secondary antibodies coupled to alkaline phosphatase (Pierce). Detection of phosphatase activity was with a chemiluminescence system (BioRad) on preflashed X-ray film (Fujifilm).

For VE-cadherin and PECAM-1, an additional method was used for the collection of the samples (Tang et al., 1993). Cells were washed twice in washing solution (10 m*M* PBS pH 7.4, 5 m*M* PMSF, and 1% aprotinin) and then harvested by scraping in TNC lysis buffer (0.01 *M* Tris-acetate pH 8.0, 0.5% Nonidet P40, 1 m*M* $CaCl_2$, 1 m*M* $MgCl_2$, 5 m*M* PMSF, 1% aprotinin, 1 m*M* benzamidine, and 0.1% trasylol). They were lysed for 45 min at 4° C and centrifuged for 30 min at 4° C, 14,000 × *g*, and stored at −80° C until use. This procedure was only applied for samples that were stained with the mouse anti–VE-cadherin from Chemicon and the mouse anti–PECAM-1 from Sigma.

γ-GT assay. The γ-GT activity was measured by a kinetic colorimetric measurement (Naftalin et al., 1969). Cells were scraped in lysis buffer pH 7.4 containing 50 m*M* HEPES, 150 m*M* NaCl, 20% glycerol, 1% Triton-X-100, 1.5 m*M* $MgCl_2$, 1 m*M* EGTA, in the presence of the following protease inhibitors: PMSF 1 m*M* (Sigma), benzamidine 0.1 m*M* (Sigma), and trasylol 0.1% (Bayer, Leverkusen, Germany). Samples were then lysed for 30 min at 4° C and stored at −80° C until the γ-GT activity was quantified with L-glutamyl-*p*-nitroanilide as substrate (Sigma kit). The protein concentration of each sample was determined by the Bradford method (BioRad).

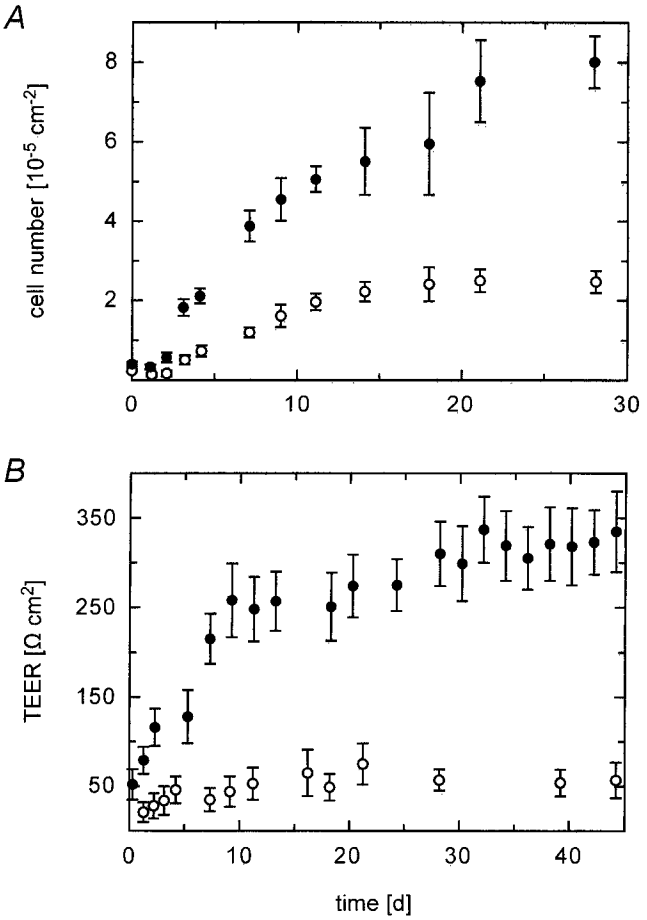


FIG. 1. Growth characteristics of ECV304 and T24 cells. Cell numbers (*A*) and TEER values (*B*) were determined as described ("Materials and Methods" section). (●) ECV304 cells; (○) T24 cells. One representative experiment for each cell line is shown with the mean ± SD determined from three culture wells.

RESULTS

Growth characteristics of ECV304 and T24 cells. Growth curves for ECV304 and T24 cells (Fig. 1*A*) were determined as described (*see* "Materials and Methods" section). Both were seeded at a density of 3–4 × 10^4 cells/cm² in M199 medium. ECV304 cells were confluent after 1 d in culture. The cell number reached a plateau of about 8 × 10^5 cells/cm² between days 16 and 21. T24 cells were confluent within 3 d, and reached a plateau of about 2.5 × 10^5 cells/cm² between days 15 and 20. The same was found when T24 cells were cultured in McCoy's 5A medium. For both cell lines, the cell numbers remained constant at least up to 28 d. TEER values were measured at various times after seeding (Fig. 1*B*). With ECV304 cells, values of about 250–350 Ωcm² were reached between days 10 and 15. They remained within this range for up to at least 28 d, whereas in T24 cells TEER values stayed at a lower level, i.e., around 50–75 Ωcm² between days 5 and 28. Both cell lines could be maintained in culture for up to 45 d without major changes in the cytoarchitecture and TEER values (data not shown).

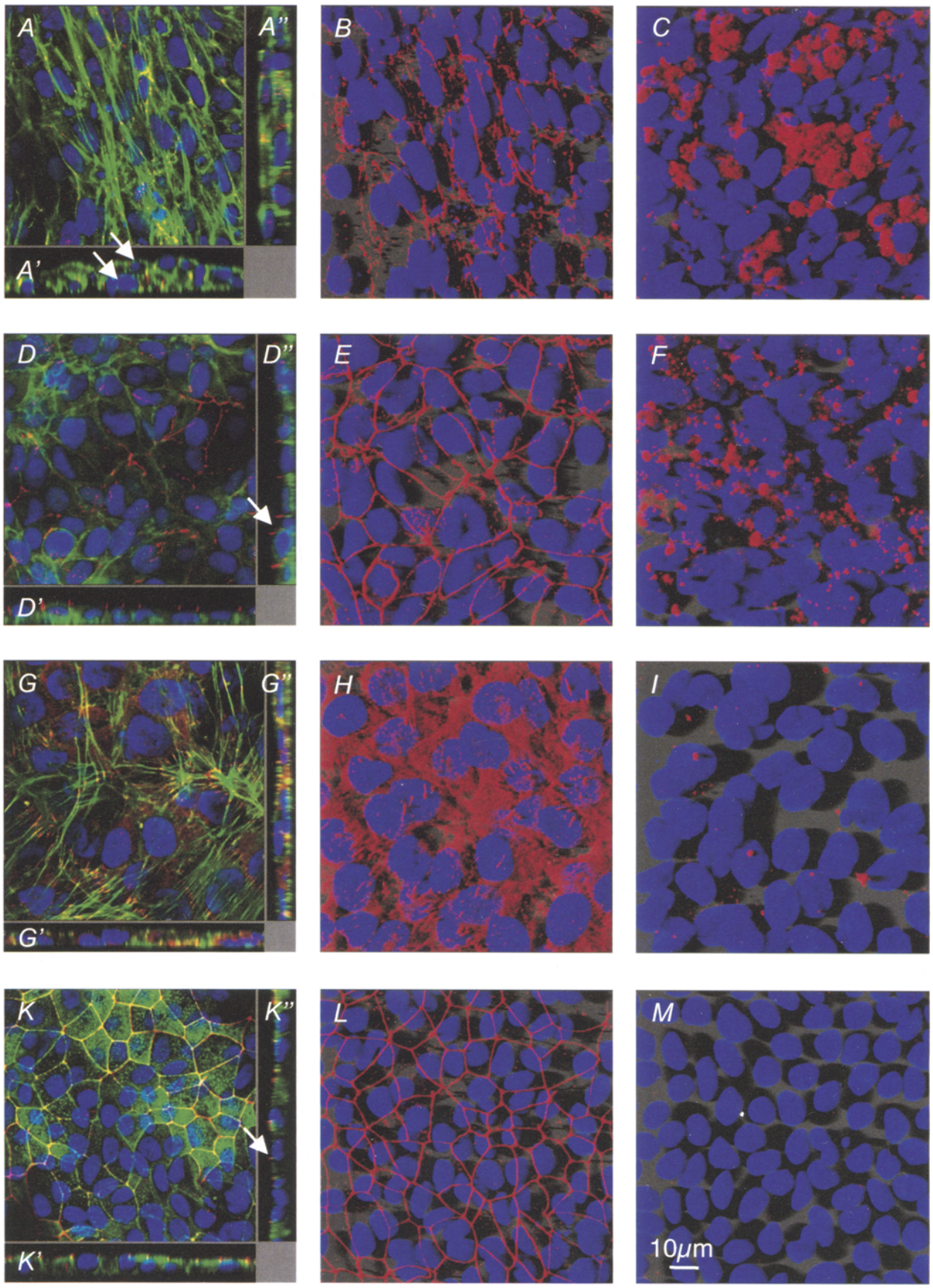
Characterization of cytoarchitecture by CLSM. In order to study the cytoarchitecture, cells were prepared for CLSM as described (*see* "Materials and Methods" section). The cell lines were investigated in the stationary phase of the growth curve, with the exception of PBMEC/C1–2 cells which detached from the insert after around 10 d in culture before reaching a plateau in the growth curve. The PBMEC/C1–2 cells showed a multilayer formation (Fig. 2*A*). This is clearly visible in the x,z- and y,z-projection, where the cell nuclei are arranged in different layers (Fig. 2*A'*, *arrows*). Strong F-actin bundles were expressed mainly along the cell borders (Fig. 2*A*), The TJ protein ZO-1 was localized at the cell borders (Fig. 2*B*), but did not form a complete network. ECV304 cells formed a monolayer (Fig. 2*D*) with TJ localized near the apical surface of the cells (Fig. 2*D''*, *arrow*). The 3D reconstruction showed that ZO-1 was expressed at the cell–cell contacts and formed a nearly complete TJ network (Fig. 2*E*). The morphology of T24 cells in turn was significantly different from that of ECV304 cells, with respect to the cytoarchitecture and localization of the ZO-1 protein. Although the T24 cells formed flat monolayers, the F-actin bundles did not line the cell borders as in ECV304 cells, but were expressed in a parallel ordered fashion instead (Fig. 2*G*). The ZO-1 protein was expressed throughout the cells (Fig. 2*H*) and in part seemed to colocalize with F-actin (Fig. 2*G*, *arrows*). The irregular arrangement throughout the cytoplasm was particularly striking in the 3D reconstruction (Fig. 2*H*). A typical flat monolayer was also found for MDCK cells (Fig. 2*K*), as seen from the arrangement of the cell nuclei (Fig. 2*K'* and *K''*). The TJs, visualized with the anti ZO-1 antibody, were localized in the upper part of the cells at the cell–cell contacts (Fig. 2*K''*, *arrow*). A regular, complete TJ network was formed (Fig. 2*L*). For occludin, another TJ-related protein, the same staining patterns as with ZO-1 were observed in all the cell lines (data not shown).

Comparative studies of DiI-Ac-LDL uptake. DiI-Ac-LDL uptake, which is a characteristic of endothelial cells, was tested in the four cell types. Highest uptake was found for PBMEC/C1–2 cells (Fig. 2*C*) followed by ECV304 cells (Fig. 2*F*). Minute traces were found in T24 cells (Fig. 2*I*), and no DiI-Ac-LDL uptake was observed in MDCK cells (Fig. 2*M*).

γ-GT activity. γ-GT activity was determined in the four cell types (Fig. 3). The activity was high in ECV304 (25.6 nmol/min/mg protein) and MDCK (28.1 nmol/min/mg protein) cells, but hardly detectable in T24 (0.7 nmol/min/mg protein) and PBMEC/C1–2 (0.8 nmol/min/mg protein) cells. Fibroblasts (BHK21), which were used as a negative control, did not show any activity (data not shown).

P-gp expression. P-gp expression in the four cell lines was compared by Western blots (Fig. 4*A*). P-gp expression was highest in PBMEC/C1–2 cells (7 d) in culture, but significant amounts were also found in MDCK (11 d) and in T24 (21 d) cells. Only traces of P-gp were present in ECV304 cells up to day 21, whereas at a later time between days 31 and 50, P-gp expression increased to a comparable level as in T24 cells.

Expression of vWF, vimentin, VE-cadherin, and PECAM-1. Expression of vWF, vimentin, VE-cadherin, and PECAM-1 was investigated by Western blots. In ECV304 cells (Fig. 4*B*), vWF could be detected already at day 4 as a clear band of around 240 kDa. This could be confirmed by the positive control, the PBMEC/C1–2 cells, which showed a strong band at a slightly lower molecular mass. In T24 cells, however, no vWF could be detected for up to 21 d in culture, the same result as was found for MDCK cells used as a negative control.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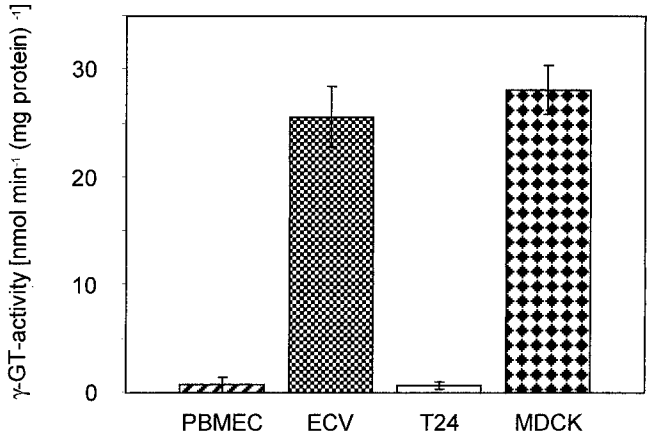

FIG. 3. γ-GT activity in PBMEC/C1–2, ECV304, T24, and MDCK cells. Cells were grown for the times indicated, and the enzyme activity measured (*see* "Materials and Methods" section): PBMEC/C1–2 (PB) cells, day 7; ECV304 and T24 cells, day 21; MDCK cells, day 11. Each *column* shows one representative experiment with the mean ± SD of triplicate measurements.

Similar results were found for vimentin (Fig. 4*C*). Strong expression was detected in PBMEC/C1–2 and ECV304 cells, even though with a slight difference in the molecular weight. In PBMEC/C1–2 cells, vimentin is localized slightly above its predicted molecular weight of 52 kDa, which can be seen in ECV304 cells. T24 cells were completely negative for vimentin, whereas in MDCK cells, some vimentin expression could be detected. Two bands were visible, from which the lower band could be assigned to vimentin according to its described molecular weight of 52 kDa. The upper band, seen also in ECV304 cells, could be either unspecific staining or a phosphorylated form of vimentin.

VE-cadherin as well as PECAM-1 was not found in any of the four cell lines investigated, although various antibodies and different methods for the collection of samples were used (data not shown).

Expression of E-cadherin, Pan-cytokeratin, and desmoglein. MDCK, T24, ECV304, and PBMEC/C1–2 cells were compared with respect to E-cadherin expression by Western blots (Fig. 4*D*) and by CLSM (Fig. 5). In Western blots, a strong band of 120 kDa for E-cadherin was detected in MDCK cells; the expression was low in T24 and PBMEC/C1–2 cells, and lowest in ECV304 cells. Similar results were found by CLSM (Fig. 5). Significant staining was present in the MDCK cells (Fig. 5*D*), particularly at the cell–cell contacts. Faint cytoplasmic staining was found in the T24 cell preparation (Fig. 5*C*). Both ECV304 (Fig. 5*B*) and PBMEC/C1–2 (Fig. 5*A*) cells showed no fluorescence at all.

Cytokeratin was stained with a Pan-cytokeratin antibody (Fig. 4*E*) which is able to detect cytokeratin 4, 5, 6, 8, 10, 13, and 18. In PBMEC/C1–2 cells, faint staining was detected for cytokeratins 8. In ECV304 and T24 cells, some of the cytokeratins were stained. Interestingly, the two cell lines did not stain the same type of cytokeratin. In ECV304 cells, a band at 45 kDa (cytokeratin 18) and a very weak band at 52 kDa (cytokeratin 8) appeared; T24 cells instead showed strong staining at 52 kDa and little staining at 45 kDa. In MDCK cells, only cytokeratin 8 was detected. The investigations with desmoglein are shown in Fig. 4*F*. As expected, desmoglein was not found in PBMEC/C1–2 cells. ECV304 and T24 cells were positive for this protein, whereas in MDCK cells, no staining could be detected.

DISCUSSION

Comparative studies on the phenotypes of the ECV304 and the T24 cell line showed significant differences (summarized in Table 1), although both have been demonstrated to have the same genotype (Dirks et al., 1999). The cell density in the stationary phase is threefold higher for ECV304 than for T24 cells. The same was found also if T24 cells were grown in McCoy's 5A medium (unpublished data). Slight differences in cell density have already been described earlier for two subclones of the T24 cell line. However, they only differed by a factor of 1.5 (Flatow et al., 1987). In accordance with published data, ECV304 as well as T24 cells form monolayers (Flatow et al., 1987; Hughes, 1996), which are, however, not as homogeneous and compact as those formed by MDCK epithelial cells (Rothen-Rutishauser et al., 1998). PBMEC/C1–2, the typical endothelial cells included in the study were not contact inhibited and grew in irregular multilayers. This behavior was also found in other transfected cells, e.g., the MDR1-MDCK cells (Hämmerle et al., 2000).

Another important difference between ECV304 and T24 cells is found in the formation and maintenance of TJ. The arrangement of TJ, as visualized in the CLSM by labeling of the TJ-related protein ZO-1, is strikingly different between the two cell types. A distinct network is present in ECV304 cells, which is in accordance with data of Kiessling et al. (1999), whereas TJ formation is rudimentary in T24 cells, although the ZO-1 protein is highly expressed throughout the cells. The same pattern was obtained for occludin, another TJ protein (unpublished data). This situation is also reflected in the TEER values: around 300 Ωcm^2 for ECV304 but only about 50 Ωcm^2 in the case of T24 cells. It has to be noted, however, that TEER values do not depend only on the TJ arrangement. For instance, ECV304 and MDCK (type II) cells exhibit similar TEER values, although the MDCK cells form a more regular network of TJ than the ECV304 cells. Also subtypes of MDCK cells (types I and II), which differ in TEER values by a factor of about 60, do not show morphological differences in TJ arrangement in the CLSM (Richardson et al., 1981; Wong, 1997).

The functional expression of γ-GT is an important feature of the

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FIG. 2. Characterization of cell cultures by CLSM. Cells were grown on filter inserts for the times indicated, and prepared for CLSM (*see* "Materials and Methods" section). (*A–C*) PBMEC/C1–2 cells, day 7; (*D–F*) ECV304 cells, day 21; (*G–I*) T24 cells, day 21; (*K–M*) MDCK cells, day 14. (*A, D, G, K*) Preparations were triple-stained for F-actin (*green*), cell nuclei (*blue*), and ZO-1 (*red*); *arrows* point to TJ (*D″, K″*), or to cell nuclei (*A′*). Colocalization of F-actin and ZO-1 results in *yellow* staining. (*B, E, H, L*) 3D reconstructions of the same areas as in (*A, D, G, K*), but only ZO-1 (*red*) and cell nuclei (*blue*) are shown. (*C, F, I, M*) DiI-Ac-LDL uptake (*see* "Materials and Methods" section), 3D reconstructions with DiI-Ac-LDL (*red*) and cell nuclei (*blue*). (*A, D, G, K*) single optical sections; (*A′, D′, G′, K′*) x,z-projections; (*A″, D″, G″, K″*) y,z-projections.

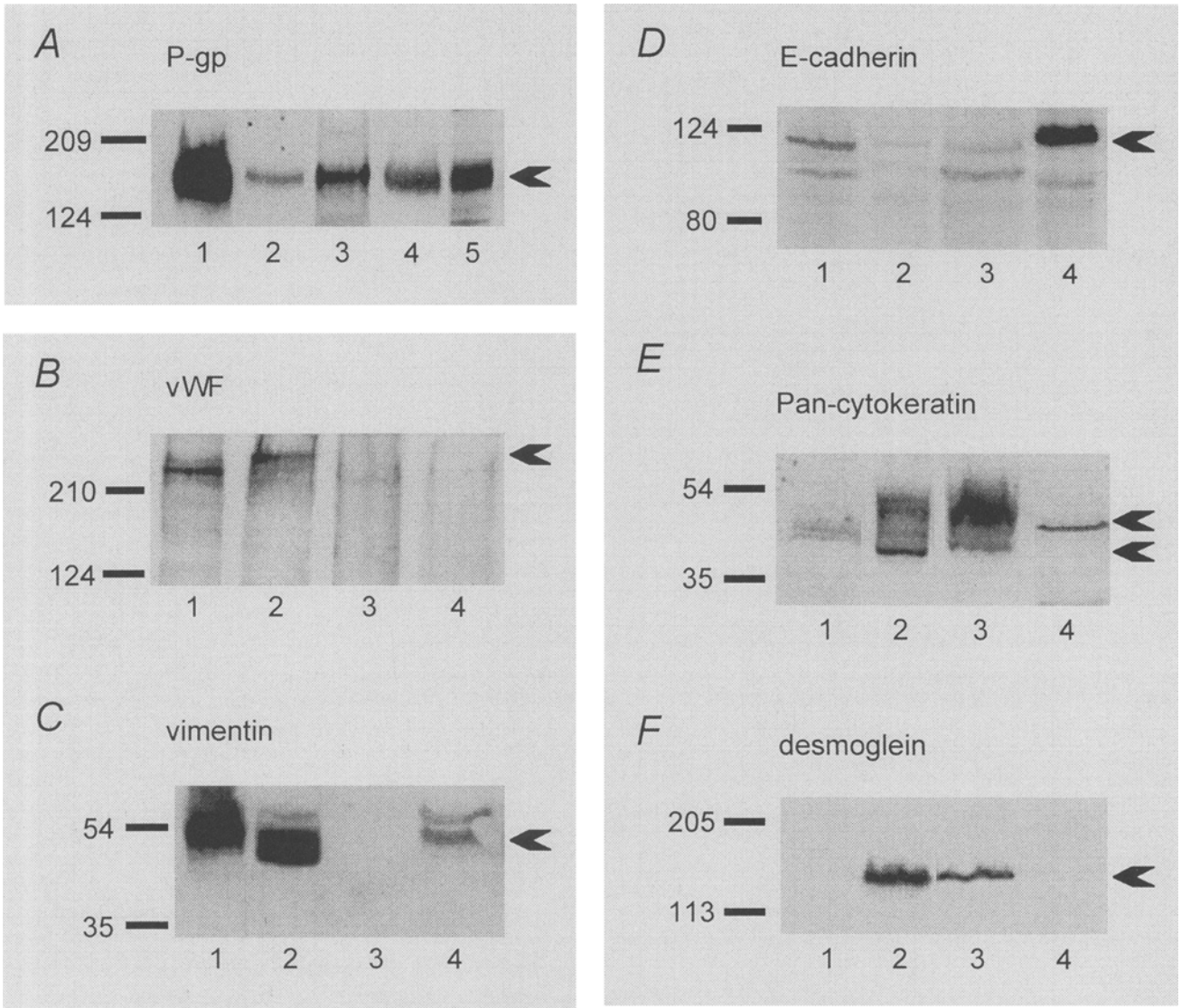

FIG. 4. Expression of P-gp, vWF, vimentin, E-cadherin, Pan-cytokeratin, and desmoglein. The expression of the indicated proteins was determined in PBMEC/C1–2, ECV304, T24, and MDCK cells by Western blots at the times indicated: (*A*) PBMEC/C1–2 cells cultured for 7 d (*lane 1*); ECV304 cells for 21 d (*lane 2*); and 50 d (*lane 3*); T24 cells for 21 d (*lane 4*) and MDCK cells for 11 d (*lane 5*). (*B–F*) PBMEC/C1–2 (day 7, *lane 1*); ECV304 (day 21, *lane 2*); T24 (day 21, *lane 3*); and MDCK (day 11, *lane 4*) except in the blot of vWF (*B*), in which the sample of ECV304 cells was already taken on day 4.

BBB because this enzyme is, among others, responsible for the degradation of xenobiotics (Frey, 1993; Commandeur et al., 1995). Even though γ-GT is generally absent in urinary bladder cells (Vanderlaan and Phares, 1981), some enzyme activity was found in T24, a carcinoma cell line. This finding is compatible with the data reported by Vanderlaan and Phares (1981), who found measurable γ-GT activity in some cultured cell lines of bladder tumors, and thus proposed the use of this activity as a marker for tumor cells. In comparison, the ECV304 cells show very high enzyme activity. It was five times the activity reported by Lechardeur et al. (1995) using the same detection kit. Unfortunately, there is no indication about the source of the ECV304 cell line in this study. The γ-GT activity found in PBMEC/C1–2 cells is significantly lower, i.e., in the same range as that reported by Teifel and Friedl (1996). The high enzyme activity measured in the ECV304 cells is in a range similar to the activity in the MDCK cells, in accordance with previously published values (Verkoelen et al., 1995).

P-gp is highly expressed in tumor cells. It is also found in the brush border of proximal tubules of the kidney, in the bile canalicular membrane of hepatocytes, in the apical membrane of mucosal cells in the intestine, and in the luminal membrane of endothelial cells at blood–tissue barrier sites (Thiebaut et al., 1987; Relling, 1996). P-gp was found in all the four cell lines tested, although to a variable extent. The highest amount was found in PBMEC/C1–2 cells cultured for 7 d, whereas for MDCK (day 11), T24, and ECV304 cells, similar amounts were found. T24 cells exhibited P-gp expression when cultured for 21 d. At that time, little P-gp was detected in ECV304 cells. Only after prolonged

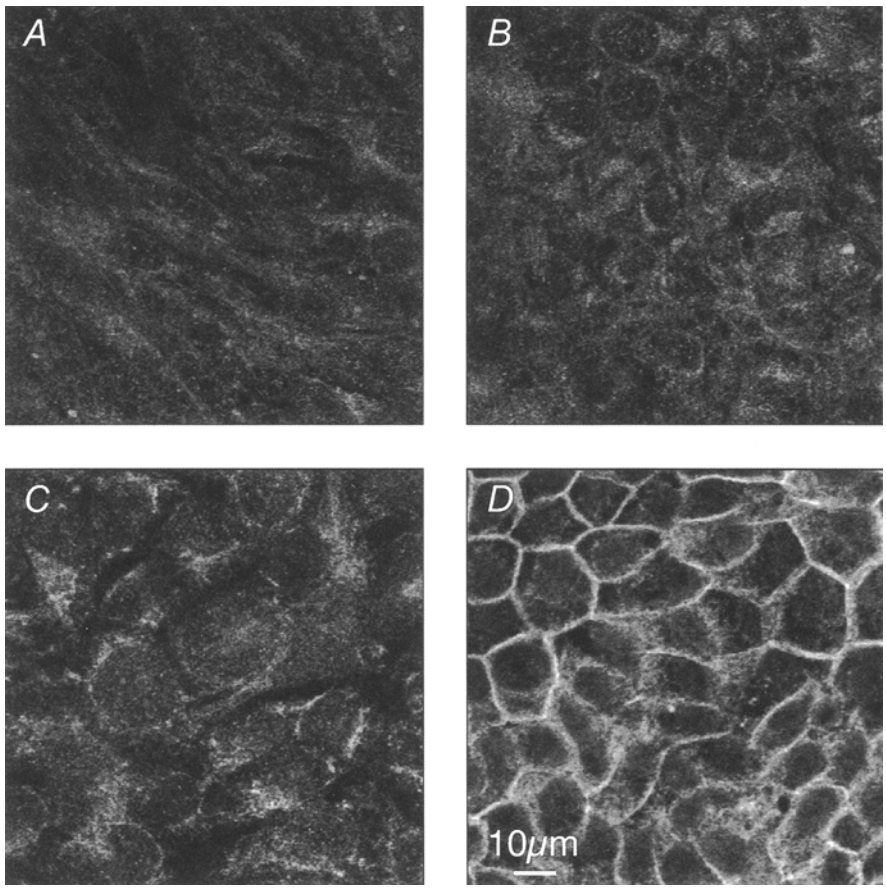

FIG. 5. Expression of E-cadherin as visualized by CLSM. Cells were cultured for the times indicated and prepared for CLSM (*see* "Materials and Methods" section). (*A*) PBMEC/C1–2, day 7; (*B*) ECV304, day 21; (*C*) T24, day 21; (*D*) MDCK, day 11. All *images* are single optical sections taken in the *middle* of the cell layers.

TABLE 1

CHARACTERISTICS OF PBMEC/C1–2, ECV304, T24, AND MDCK CELLS IN STATIONARY PHASE (SUMMARY)[a]

	PBMEC/C1-2	ECV304	T24	MDCK
General features				
cell number [cells/cm²]	no stationary phase	**8 × 10⁵**	**2.5 × 10⁵**	5 × 10⁵
TEER [Ωcm²]	150 ± 30	**300 ± 50**	**50 ± 20**	250 ± 30
TJ network (ZO1)	+	**++**	**—**	+++
P-gp	++++	**+ (21 d)** **++ (50 d)**	**++ (21 d)**	+++
γ-GT [nmol/min/mg protein]	0.76 ± 0.31	**25.63 ± 2.78**	**0.7 ± 0.37**	28.13 ± 2.26
Endothelial markers				
Ac-LDL uptake	+++	**++**	**+**	—
vWF	+++	**+++**	**—**	—
vimentin	+++	**+++**	**—**	+
VE-cadherin	—	—	—	—
PECAM-1	—	—	—	—
Epithelial markers				
E-cadherin	+	+	+	+++
cytokeratin 8	+	**+**	**+++**	+
cytokeratin 18	—	**++**	**+**	—
desmoglein	—	++	+	—

[a]Experiments were performed as described in "Materials and Methods." Differences between ECV304 and T24 cells are in *boldface*.

culturing (30–50 d), comparable amounts of P-gp protein were present. This difference could be explained by the different growth behavior of the two cell lines. In T24 cells, the stationary phase of the growth curve is reached between days 10 and 12 after seeding (seeding density: 5×10^4 cells/cm^2), whereas with ECV304 cells, this plateau is only attained after about 21 d in culture (seeding density: 5×10^4 cells/cm^2). Therefore, the differentiation of ECV304 cells, e.g., with regard to transporters, is delayed when compared with T24 cells. A similar situation can be found in Caco-2 cells which need at least 3 wk to build up their specific transporter systems (Hosoya et al., 1996).

The uptake of DiI-Ac-LDL is regarded as a characteristic feature of endothelial cells (Hewett and Murray, 1993), and therefore high uptake of DiI-Ac-LDL was expected for the PBMEC/C1–2 cells. This was indeed the case. Uptake was also found in ECV304 cells, although to a lesser extent than in PBMEC/C1–2 cells. No significant uptake was registered in the T24 cell line and in MDCK cells. DiI-Ac-LDL uptake in ECV304 cells has already been described though to a lesser extent than in freshly isolated human umbilical vein endothelial cells (Hughes, 1996). Furthermore, the vWF is a widely used endothelial marker. It was shown to be expressed in endothelial cells, megakaryocytes, and platelets, where it is concentrated in the Weibel-Palade bodies, presumable storage and/or processing vesicles for this protein (Wagner et al., 1982). We could identify this marker in the ECV304 and PBMEC/C1–2 cells. The finding for ECV304 cells is in agreement with Hughes (1996). Both, DiI-Ac-LDL uptake and the vWF have been reported to be present in mesothelial cells (Chung-Welch et al., 1997). Mesothelial cells are similar to endothelial cells, with which they share many morphological and functional properties. However, vWF expression and DiI-Ac-LDL uptake are not found in epithelial cells and never have been described in bladder cells. An additional hint for an endothelial phenotype in ECV304 cells is the finding that they organize into an extensive network of capillary tube-like structures within 1 d when cultured on Matrigel, a laminin-based support (Hughes, 1996). No comparable studies have been performed with T24 cells.

Two other endothelial markers, VE-cadherin and PECAM-1, could not be detected in any of the studied cell lines, although various antibodies were used (two for each protein). The fact that in PBMEC/C1–2 cells too no expression of VE-cadherin or PECAM-1 could be detected may be interpreted to indicate that the antibodies, which were raised against humans or mice, are species-specific and do not bind to the porcine forms. This argument does not, however, hold for ECV304, a human cell line. These cells have already been described to be negative for PECAM-1 and VE-cadherin (Hughes, 1996; Kim et al., 1998; Kiessling et al., 1999).

Vimentin is mainly found in cells of mesenchymal origin, such as fibroblasts, some leukocytes, and endothelial cells, but it was also found in epithelial cells, such as mesothelial cells and the human kidney (Holthofer et al., 1984; Chung-Welch et al., 1997). Our data perfectly agree with these findings. PBMEC/C1–2 cells strongly expressed vimentin. Strong expression was also found in ECV304 cells, whereas T24 cells did not express it at all. The weak expression found in MDCK cells is in accordance with the published data (Stuart et al., 1994). Staining for Pan-cytokeratin revealed cytokeratin expression in all the four cell lines. Even in PBMEC/C1–2 cells, faint staining was observed. Cytokeratin as well is not restricted to epithelial tissue. Cytokeratins of types 8, 18, and 19 are usually expressed in simple epithelia. They have also been found in lymph nodes, peripheral blood cells, skin fibroblasts, freshly isolated endothelial cells (Traweek et al., 1993), human aortic smooth muscle cells (Glukhova et al., 1991), mesothelial cells (Connell and Rheinwald, 1983), and pulmonary microvessel endothelial cells (Alexander et al., 1991) as well as subsynovial capillary blood vessels (Stosiek et al., 1990). The coexpression of vimentin and cytokeratin documented in this study has been described before (Schmid et al., 1983; Holthofer et al., 1984; Czernobilsky et al., 1985).

E-cadherin was used as a typical epithelial cell marker. Significant amounts of E-cadherin were detected in the MDCK cells by CLSM as well as by Western blots. The ECV304, T24, and PBMEC/C1–2 cells showed a faint staining in Western blots, but only background staining in the CLSM. The results for ECV304 are in contrast with Kiessling et al. (1999) who clearly showed E-cadherin staining for ECV304 cells in Western blots, and also reported focal areas of lateral membrane staining by immunofluorescence in the CLSM. It cannot be excluded that this discrepancy could be caused by the different antibodies used. However, the findings of strong staining in MDCK cells, as well as the positive staining in the other cell lines, make this explanation unlikely. Another explanation could be the use of different subclones of the same cell line. Kiessling et al. (1999) worked with ECV304 cells of the ECACC culture collection, whereas the ECV304 cells in this work stem from the ATCC cell bank. Scism et al. (1999) reported significant differences between the ECV304 cells of the ECACC and ATCC collections, respectively; in particular, differences in TEER and sucrose permeability were observed. A very important difference is that ECV304 cells from the ATCC are not inducible for BBB characteristics by astrocyte or glioma coculture, whereas the cells from ECACC can be induced. In view of these findings, differences in the protein pattern of the two subclones could be expected as well. Systematic studies have yet to be performed, but comparing the markers used in this study and in that of Hughes (1996), some indications can already be found regarding the expression of vWF and of cytokeratin types 6 and 10.

The weak signal for E-cadherin in T24 cells is consistent with the previously published loss of E-cadherin expression in T24 cells as compared with normal urinary bladder cells (Wakatsuki et al., 1996; Imao et al., 1999). The authors suggested an inverse correlation between E-cadherin expression and clinical aggressiveness of tumors. It is interesting to note that E-cadherin has also been found in primary brain endothelial cells (Rubin et al., 1991; Pal et al., 1997). High E-cadherin expression, which is typical for an epithelial phenotype, such as MDCK cells, is neither found in ECV304 nor in T24 cells.

Desmosomes are structures typically found in epithelial cells, whereas in endothelial cells, syndesmos, desmosomal-like structures, were described. Desmoglein and desmoplakin expression was demonstrated in ECV304 cells (Kiessling et al., 1999). Our results confirmed this finding for desmoglein which was found in ECV304 and T24 cells. No staining has been performed with desmoplakin because this protein is a member of syndesmos as well and therefore cannot be used to distinguish between an epithelial or an endothelial feature.

To sum up, although the ECV304 and T24 cell lines have an identical genotype, they exhibit clear phenotypic differences. They differ not only in their TEER, growth behavior, and cytoarchitecture,

but also in the expression pattern of endothelial and epithelial markers, respectively. Neither the ECV304 cell line nor the T24 cell line showed a complete endothelial phenotype. However, T24 cells were lacking in important endothelial features, such as significant LDL uptake, expression of vWF and vimentin, or the formation of a complete TJ network, whereas ECV304 cells clearly exhibited these endothelial traits. Regarding all available data, we conclude that at the present moment, with a paucity of endothelial cell lines available, the ECV304 cell line is still a suitable endothelial model. ECV304 cells from ATCC exhibit endothelial characteristics and a nearly complete TJ network without coculture with astrocytes or astrocyte supernatants.

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