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Expression of Claudin-1, Claudin-3 and Claudin-5 in human blood–brain barrier mimicking cell line ECV304 is inducible by glioma-conditioned media

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ABSTRACT

Up to now no standard cell culture model of the blood–brain barrier is available. However, several models based on primary cells or continuous cell lines have been characterized and described in respect of different applications. One of the most important characteristics of the blood–brain barrier is the restriction of paracellular transport, respectively its tightness. Human cell line ECV304 is one of the promising continuous cell lines for blood–brain barrier modelling due to two reasons: on the one hand the cells are able to form significant tighter layers than most of the other cell lines used and on the other hand several properties of the blood–brain barrier are inducible by using glioma-conditioned medium. Claudins are transmembranal proteins which form the backbone of the tight junctions at the blood–brain barrier. We have investigated the presence and inducibility of the expression of Claudin-1, Claudin-3 and Claudin-5 using immunofluorescence microscopy. For the first time this study proves the presence of Claudin-1, Claudin-3 and Claudin-5 in ECV304 (obtained from ECACC) cell layers and the inducibility of their expression by glioma-conditioned media.

The blood–brain barrier (BBB) maintains the homeostasis of the brain microenvironment which is crucial for neuronal activity and function. Brain microvascular endothelial cells (BMEC) that constitute the BBB are responsible for the transport of nutrients to neurons and the clearance of potentially toxic substances from the brain. Unlike the peripheral endothelium, BMEC are characterized by the presence of tight intercellular junctions, minimal pinocytotic activity and the absence of fenestrations [20]. Bands of tight junctions (zonula occludens) between adjacent endothelial cells restrict the paracellular pathway and effectively prevent the passage of polar, hydrophilic drugs through the endothelial cell layer. The resulting distinct tightness of cerebral capillary junctions is reflected in high transendothelial electrical resistances (TEER). Typically, electrical resistances of about 2000 $\Omega\,cm^2$ are observed *in vivo* in pial microvessels on the surface of the brain [4] as compared to 1–3 $\Omega\,cm^2$ in mesenteric capillaries [5].

Several *in vitro* cell culture models of the blood–brain barrier have been characterized [7,15]. However, no standard model has been developed until now. For high-throughput purposes and screening processes in drug discovery and development the application of continuous cell lines is preferred. Cell line cultures can be expanded at will which drastically reduces cost and labor, and they are easier to culture in comparison to more time-consuming primary brain endothelial cells [6]. Also, the phenotypic characteristics are stable over a longer period improving reproducibility of data. One disadvantage of most continuous cell lines for BBB modelling is their insufficient tightness in comparison to primary cells. Thus, the search for tight continuous cell lines exhibiting BBB properties remains very important to enable meaningful BBB drug screening.

Human cell line ECV304 is one of the most promising continuous cell lines for blood–brain barrier modelling together with the murine cell line cEND and the human cell line hCMEC/D3 [11,12]. Doubt has been raised over the use of ECV304 for BBB studies and whether these cells are endothelial [3,9]. However, in contrast to ECV304 cells from ATCC several studies have shown that BBB properties were inducible when ECV304 from ECACC were co-cultured with astrocytes or glioma cell line C6 [8,10,18,24]. Because of the fact that inducibility of BBB properties is one of the most important features for BBB models, ECV304 cells from ECACC were chosen for our studies. Furthermore, ECV304 layers are able to form significantly tighter junctions as reflected in higher TEER values than other BBB cell lines such as RBE4, b.End3, b.End5, RBEC and EaHy929 [30,31,35]. Cell line cEND can form a distinct barrier reflected in high TEER values but it is from murine origin [12]. Förster et al. [11]

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optimized a cultivation protocol in order to achieve higher TEER values of hCMEC/D3 layers by adding hydrocortisone in a serum-poor medium. However, tightness of hCMEC/D3 layers was not increasible by astrocytic factors [33]. Thus, cell line ECV304 is currently the only human cell line for BBB modelling, which responds to astrocytic factors and can build up a tight barrier.

In this context, we have recently performed a detailed tightness characterization of ECV304 layers grown in different media with and without soluble factors derived from glioma cell line C6 [27]. Within these studies the tightness of the cell layers was investigated on the functional (TEER, permeability of paracellular marker APTS-dextran) as well as on the biomolecular level. Therein, immunofluorescence microscopy revealed that the expression of tight junctional protein ZO-1 was increased by adding glioma-conditioned medium to the basal growth medium.

To date, presence and/or inducibility of the expression of tight junctional proteins by glioma derived soluble factors were only reported for ZO-1 and Occludin of cell line ECV304 obtained from ECACC [19,21,27]. However, tight junctional proteins called Claudins are known to form the backbone of tight junctions of the BBB. The presence of Claudin-1, Claudin-3 and Claudin-5 were reported and identified as major components of tight junction strands at the blood–brain barrier [17,25,26,32,34]. Consequently, the aim of the presented report was to investigate whether ECV304 cell layers express tight junctional proteins Claudin-1, Claudin-3 and Claudin-5 and whether their expression can be increased by glioma-conditioned medium.

For this reason, ECV304 cells obtained from ECACC were cultured in three different media: basal ECV304 medium (ECV304 cell passage 142–148), GCM of ECV304 medium (ECV304 cell passage 143–147) and PBMEC medium (ECV304 cell passage 142–177). The basal ECV304 medium was made of M199 with 10% (v/v) fetal calf serum, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B. The GCM (glioma-conditioned medium) of ECV304 medium was obtained by culturing glioma C6 cells at passages 18–50 in 175 cm^2 gelatine-coated tissue culture flasks (Greiner Bio-One GmbH, Frickenhausen, Germany) in ECV304 medium and collecting the supernatant (25 mL per day). C6 cells derived from rat glioma [2] were obtained from the German Cancer Research Center Heidelberg (DKFZ Heidelberg, Germany). The PBMEC medium (50% C6 medium and 50% GCM of C6 medium) was used, since a continuous tight network of ZO-1 was shown for ECV304 layers grown in this medium [27]. The C6 medium consisted of IF medium (1:1 mixture of IMDM and Ham's F-12) with 7.5% (v/v) newborn calf serum, 7 mM L-glutamine, 5 μg/mL transferrin, 0.5 U/mL heparin, 100 U/mL penicillin, 100 μg/mL streptomycin, and 0.25 μg/mL amphotericin B. GCM of C6 medium was produced as described above using glioma C6 cells. Culture conditions for all cells were set to 96% humidity in an incubator with 5% CO_2/95% air atmosphere. Cells were subcultivated by trypsinization every 3–5 days. For immunofluoresence microscopy 50,000 ECV304 cells/cm^2 were seeded onto cover slips (diameter 18 mm, Assistent, Germany) in a 12-well plate (surface area 3.8 cm^2, Iwaki, Japan) using 2 mL of the respective medium.

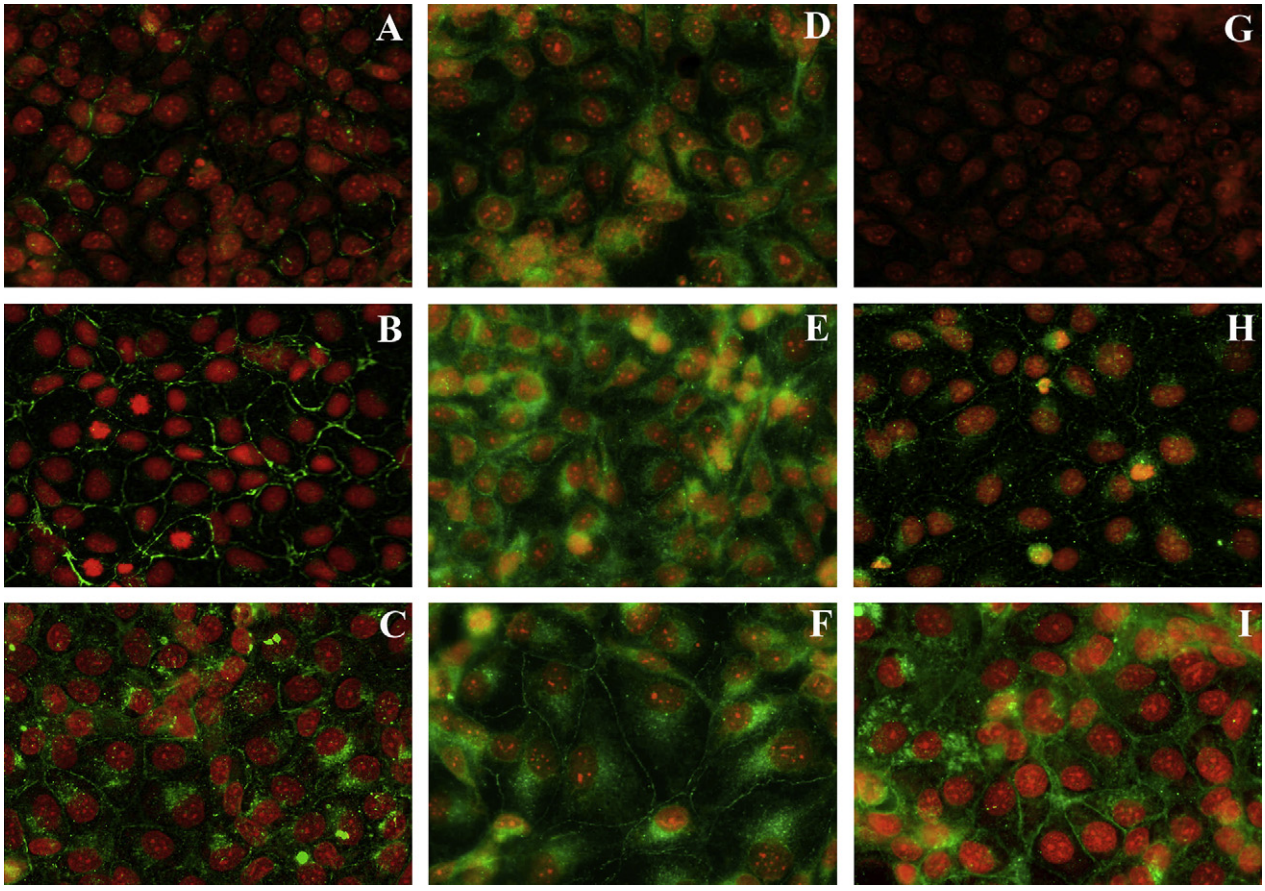

Fig. 1. Immunofluorescence images of Claudin-1 of ECV304 cells cultured in ECV304 medium (A), ACM of ECV304 medium (B) and PBMEC medium (C), of Claudin-3 of ECV304 cells grown in ECV304 medium (D), ACM of ECV304 medium (E) and PBMEC medium (F), and of Occludin of ECV304 cells grown in ECV304 medium (G), ACM of ECV304 medium (H) and PBMEC medium (I). Green tight junctional proteins are localized at cell boarders and perinuclearly; red cell nuclei were stained with propidium iodide. Images were generated with polyclonal primary antibodies from Santa Cruz and Zymed®. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)