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Research article

Type of culture medium determines properties of cultivated retinal endothelial cells: induction of substantial phenotypic conversion by standard DMEM

Catharina Busch [a,1], Matus Rehak [a,1], Margrit Hollborn [a], Peter Wiedemann [a], Gerhard K. Lang [b], Gabriele E. Lang [b], Armin Wolf [b], Heidrun L. Deissler [b,*]

[a] Department of Ophthalmology, University Hospital Leipzig, Leipzig, Germany
[b] Department of Ophthalmology, University of Ulm Hospital, Ulm, Germany

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ABSTRACT

Contradictory behavior of microvascular retinal endothelial cells (REC) - a reliable in vitro model to study retinal diseases - have recently been reported which might result from cultivating the cells in standard DMEM not optimized for this cell type. Therefore, we studied DMEM's effects on phenotype and behavior of immortalized bovine REC. Cells were cultivated in endothelial cell growth medium (ECGM) until a confluent monolayer was reached and then further kept for 1–4 days in ECGM, DMEM, or mixes thereof all supplemented with 5% fetal bovine serum, endothelial cell growth supplement, 90 μg/ml heparin, and 100 nM hydrocortisone. Within hours of cultivation in DMEM, the cell index – measured to assess the cell layer's barrier function - dropped to ~5% of the initial value and only slowly recovered, not only accompanied by stronger expression of HSP70 mRNA and secretion of interleukin-6, but also by lower expressions of tight junction proteins claudin-5, claudin-1 or of the marker of cell type conversion caveolin-1. Altered subcellular localizations of EC-typic claudin-5, vascular endothelial cadherin and von Willebrand factor were also observed. Taken together, all experiments with (retinal) EC cultivated in common DMEM need to be interpreted very cautiously and should at least include phenotypic validation.

1. Introduction

Endothelial cells (EC) isolated from different vascular beds are the basis of valuable *in vitro* models proved to be useful to investigate important physiological and pathological processes. Of their different types, retinal microvascular EC (REC) isolated from various species have been widely used in research to better understand the pathogeneses of ocular diseases of high socio-economic relevance like macular edema secondary to diabetic retinopathy or retinal vein occlusion [1, 2, 3, 4, 5, 6, 7]. It is of particular interest and of relevance to the interpretation of experimental data that primary or immortalized REC originating from different species (e.g. *homo sapiens*, *bos taurus*, *sus scrofa*) behave in very similar if not identical ways, but in many regards very differently from EC isolated from large vessels [3, 4, 5, 6, 7, 8, 9, 10, 11, 12]. This is a consequence of the distinct gene expression patterns of microvascular and macrovascular EC [13]. However, more and more contradictory or inconsistent results have been published during recent years: Deissler et al. found that placenta growth factor (PlGF) did not elevate permeability of confluent REC, whereas Huang et al. observed such an effect [5, 14]. REC express the mRNA(s) coding for the several splice variants of vascular endothelial growth factor (VEGF-A), but the cells seem not to produce or secrete the corresponding proteins - at least when having formed a confluent monolayer [6, 15, 16]. Stewart et al. showed that elevated glucose levels inhibited proliferation of REC, but Wang et al. found that their proliferation was stimulated under such conditions [17, 18].

The most obvious differences between these pairs of studies were the different cell culture media used, i.e. cell culture medium composed to meet the special requirements of microvascular EC versus the commonly used Dulbecco's modified Eagle's medium [19, 20, 21].

In order to reveal reasons of the striking inconsistencies of these results, we investigated if cultivating the well-established cell line of

* Corresponding author.
E-mail address: heidrun.deissler@uniklinik-ulm.de (H.L. Deissler).
[1] contributed equally.

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immortalized microvascular EC of the bovine retina (iBREC) in suboptimal standard medium (i.e. Dulbecco's modified Eagle's medium) affected the distinct characteristics and behavior of the cells [8]. Assessed properties included phenotype, metabolic activity, and barrier stability. We also studied expression or subcellular localization of proteins typical of ECs and associated with their specialized function: von Willebrand factor (vWF), the adherens junction (AJ)-protein vascular endothelial cadherin (VEcadherin), tight junction (TJ)-proteins claudin-5 and claudin-1, transport protein caveolin-1, and VEGF-A [3, 4, 5, 6, 7, 8, 12, 22, 23, 24, 25, 26]. In addition, expression of mRNA encoding the heat-shock protein 70 (HSP70) and secretion of interleukin-6 (IL-6) was measured as indicators of cellular stress response [27, 28].

2. Material and methods

2.1. Cultivation of iBREC

Telomerase-immortalized microvascular endothelial cells from bovine retina (iBREC) were used between passages 25 and 55 (counting from the stage of primary culture) for which stable expression of investigated proteins and marker proteins typical for EC has been confirmed [4, 5, 8]. The cells were cultivated in Endothelial Cell Growth Medium MV (ECGM-MV, C-22120, Promocell, Heidelberg, Germany) containing 1 g/l glucose and supplemented with 100 nM hydrocortisone (Promocell), 10 ng/ml human epidermal growth factor (hEGF; Promocell), 0.4% endothelial cell growth supplement/H (ECGS/H; bovine hypothalamic extract with 22.5 mg/ml heparin resulting in a final heparin concentration of 90 µg/ml in supplemented ECGM-MV; Promocell), 5% fetal bovine serum (FBS; Promocell) and 300 µg/ml geneticin (ThermoFisher Scientific, Langenselbold, Germany) on fibronectin-coated (50 µg/ml; BD Biosciences, Corning, Amsterdam, The Netherlands) surfaces. Kept at 37 °C in a humidified atmosphere with 5% CO_2, the cells went through passaging every three days [4, 5, 8]. All experiments were performed with confluent monolayers of iBREC established three to four days after seeding and cultivation in ECGM-MV. In addition to routinely confirming expression of proteins typical for EC, we also recorded iBREC's characteristic proliferation profile by electric cell-substrate impedance measurements using the microelectronic biosensor system for cell-based assays xCELLigence RTCA DP (Acea, OLS, Bremen, Germany) (see 2.2) [6].

The cell culture media compared in this investigation, i.e. ECGM-MV or Dulbecco's Modified Eagle's Medium (21885025, ThermoFisher Scientific), both contained 1 g/l glucose and were used in all experiments after supplementation with 0.4% ECGS/H, 100 nM hydrocortisone, 5% FBS and 300 µg/ml geneticin, resulting in complete media referred to as ECGM or DMEM, respectively. hEGF does not enhance the stability of the barrier formed by iBREC and was therefore not a component of the investigated cell culture media [16]. To avoid stress responses of the cells to pH or temperature fluctuations, fresh cell culture medium was used only after sufficient pre-equilibration.

2.2. Cell index measurement

As an indicator of iBREC's barrier stability, adhesion and vitality, properties potentially dependent on the type of cell culture medium, the electric cell-substrate impedance was measured continuously with the microelectronic biosensor system for cell-based assays xCELLigence RTCA DP [6, 29, 30]. Impedance was measured between gold electrodes in individual wells of an E-Plate and calculated as the unit-free parameter cell index $CI=(Z_i-Z_0)/15\ \Omega$ (RTCA Software 2.0, Acea). In this formula, Z_i is the impedance measured at an individual time point and Z_0 the impedance read at the start of the experiment [29]. Cells were cultivated in wells of an E-Plate 16 PET (Ø 5 mm per well, 16 wells per plate; Agilent, OLS) of which three plates were always measured in parallel to analyze the CI in 48 individual wells. iBREC ($\sim 2 \times 10^4$ cells per fibronectin-coated well) were seeded in 200 µl ECGM-MV and cultivated

Table 1. Target-specific and detection antibodies used[a].

Target	Host and Type	Source	Working Concentrations
actin	mouse, monoclonal	clone 5J11, ab190301, abcam (Cambridge, United Kingdom), or NBP2-25142, bio-techne (Wiesbaden, Germany)	WB: 200 ng/ml
caveolin-1	rabbit, polyclonal	ab2910, abcam	WB: 20 ng/ml
claudin-1	rabbit, polyclonal	JAY.8, 51–9000,ThermoFisher Scientific	WB: 100 ng/ml
claudin-5	rabbit, polyclonal	Z43.JK, 34–1600,ThermoFisher Scientific	WB: 100 ng/ml IF: 2.5 µg/ml
VEcadherin	rabbit, polyclonal	2158S,Cell Signaling Technology B.V. (Frankfurt, Germany)	WB: 1:1000 IF: 1:100
vWF	rabbit, polyclonal	AP23565PU-N,Origene (Herford, Germany)	IF: 1:500
whole IgG, rabbit	goat, polyclonal, coupled to HRP	170-5046, bio-rad (Munich, Germany),	WB: 1:30000
whole IgG, mouse	goat, polyclonal, coupled to HRP	170-5047, bio-rad	WB: 1:30000
IgG, H + L chains, rabbit	goat, F (ab')$_2$ fragments, coupled to AlexaFluor595	A11072,ThermoFisher Scientific	IF: 1:500

[a] IF: immunofluorescence, WB: Western blot.

until a confluent monolayer was reached three to four days later as indicated by a stable CI (see Figure 2A, CI measurements every 15 min) [6]. Cell culture medium was completely removed without the cell monolayer being impaired and 200 µl of ECGM, DMEM or mixtures thereof were added per well; the CI was then determined every 2 min for 90 min, followed by measurements every 5 min for 62.5 h and then every 15 min until completion of the experiment. Recorded CI values obtained from at least six wells per tested condition are either presented as absolute values or were normalized in relation to those measured immediately before the cell culture media were exchanged (RTCA Software 2.0). The results were converted to graphs showing means and standard deviations using Graph Pad Prism 6 (Graph Pad, San Diego, USA).

2.3. Cell viability assay

To assess cell viability, enzymatic conversion of 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT; SERVA, Heidelberg, Germany) by NAD(P)H-dependent oxidoreductases was determined. iBREC (6.5×10^3 cells) were seeded in a fibronectin-coated well of a 96-well flat-bottom plate and cultivated in ECGM-MV until cells had formed a confluent monolayer. After 2 h, 20 h or 68 h of cultivation in the different cell culture media, respectively, 10 µl MTT solution (5 mg/ml) were added per well and cells were incubated for additional four hours before the supernatants were removed from the cells. Then 100 µl dimethyl sulfoxide were added to each well and each of the resulting cell suspensions was agitated until complete solubilization, before absorbance at 570 nm was measured with a Spectra Max 50 ELISA reader (Molecular Devices, Sunnyvale, CA, USA). To compare independent experiments, values were normalized in relation to those obtained from similarly processed control cells cultivated in ECGM.

2.4. Preparation of protein extracts and western blot analyses

Confluent iBREC in fibronectin-coated cell culture flasks (75 cm^2, Sarstedt, Nuembrecht, Germany) were further cultivated in ECGM, DMEM or mixtures of these media as described above. Cells harvested one day, three or four days later were washed in phosphate buffered saline (PBS; ThermoFisher Scientific) before they were resuspended in 100 µl lysis buffer 17 (biotechne)/10^6 cells supplemented with 5 µg/ml aprotinin, 20 µg/ml pepstatin, 5 µg/ml leupeptin (all from Roche Diagnostics, Mannheim, Germany) and phosphatase inhibitor cocktail 2 (1:200; Sigma Aldrich, Deisenhofen, Germany). After incubation on ice for 30 min under agitation, the suspensions were centrifuged (30 min, 21200 x g, 4 °C) and the protein concentrations of the supernatants determined using the Pierce ThermoFisher Scientific BCA Protein Assay Kit according to the manufacturers' instructions. Portions of these lysates (~10 µg protein from ~3×10^5 iBREC, diluted in 4x Laemmli Sample Buffer (bio-rad) supplemented with 100 mM 1,4-dithiothreitol) were separated by electrophoresis in a 4–20% Mini Protean TGX Precast Gel (bio-rad) using TGS buffer (192 mM glycine, 25 mM Tris, 0.1% sodium dodecyl sulphate (SDS), pH 8.4) for 40 min at 200 V and 40–80 mA. Separated proteins were then transferred by electroblotting in TGM buffer (same as TGS buffer, but without SDS and with 20% methanol) to a polyvinylidene difluoride membrane (bio-rad) for 100 min at 160 mA and 12–70 V using a PerfectBlue Semi-Dry-Blotter (peqlab, VWR, Darmstadt, Germany) [6, 7, 16]. Membranes were then incubated at room temperature for 90 min or over night at 4 °C in blocking solution (0.5% blocking reagent (Roche Diagnostics) dissolved in 0.1% Tween-20 (bio-rad)/PBS devoid of Ca^{2+}- or Mg^{2+}-ions (PBSd; ThermoFisher Scientific)). Primary and secondary antibodies (for working concentrations see Table 1) were always diluted in 0.1% blocking reagent/0.1% Tween-20/PBSd. For detection of the proteins of interest, membranes were placed in the tablets of the automatic Blotbot system FreedomRocker (New Advance, Biostep, Burkhardtsdorf, Germany) and incubated at room temperature with primary antibodies and the appropriate IgG horseradish peroxidase conjugates under gentle rocking for 90 min or 30 min, respectively; after each incubation step, membranes were washed three times for 5 min with 0.1% Tween-20/PBSd. Membranes were exposed to the Pierce ECL Plus Western blotting substrate (ThermoFisher Scientific) according to the manufacturer's instruction and chemiluminescence signals were visualized by direct scanning with the imaging system Fusion Pulse TS (Vilbert Lourmat, VWR) [6, 7, 16]. To quantify chemiluminescence signals, the peak volumes of the corresponding bands were determined with Evolution Capt software (Vilbert Lourmat) and set in relation to those of actin-specific bands resulting from the same sample [6, 7, 16]. Values were normalized in relation to those obtained from similarly processed control cells cultivated in ECGM.

To determine presence of multiple antigens in parallel, the protein-bound membrane was cut in half just below the size marker of 35 kDa. After blocking, the upper half of the membrane (35 kDa–250 kDa) was first exposed to antibodies directed against actin, the lower half (10 kDa–35 kDa) to claudin-1-specific antibodies followed by incubation with the appropriate secondary antibodies and detection of antibody-specific signals as described above. Membranes were then exposed to Restore Plus Western Blot Stripping Buffer (ThermoFisher Scientific) for 45 min at room temperature to remove bound antibodies. After washing the membranes three times for 5 min with 0.1% Tween-20/PBSd followed by blocking, the upper half of the membrane was exposed to antibodies against VEcadherin, the lower half to claudin-5-specific antibodies as described above. To determine the presence of caveolin-1, bound antibodies were removed from the lower half of the membrane which was then exposed to caveolin-1-specific antibodies as described above.

2.5. Immunofluorescence staining

Immunofluorescence staining of confluent iBREC pre-cultivated on fibronectin-coated two-chamber slides (x-well PCA Tissue Culture Chambers; Sarstedt) and then kept in ECGM or DMEM was performed to detect proteins of interest (i.e. claudin-5, vWF or VEcadherin) and to determine their subcellular localization [6, 30]. In detail, cells were fixated in methanol at -20 °C for 10 min, washed in PBSd three times for 5 min before they were incubated in 1% NP-40 substitute (Roche Diagnostics)/PBSd for 10 min, all carried out at room temperature. All incubation steps were done at room temperature in the dark, always followed by washing steps consisting of three consecutive incubations in PBSd for 5 min. Slides were then blocked in 10% ImmunoBlock (Roth, Karlsruhe, Germany)/PBSd for one hour, exposed to the primary antibody for one hour and subsequently to the appropriate secondary antibody for 30 min before - for examination by fluorescence microscopy (DM4000B, FW4000, Leica, Wetzlar, Germany) - the cells were embedded in ProLong Gold/Diamond Antifade Mountant (ThermoFisher Scientific) which contains 4',6-diamidino-2-phenylindole (DAPI) to stain the nuclei. Primary and secondary antibodies (for working dilutions/concentrations see Table 1) were always diluted in 1% ImmunoBlock/PBSd. Nuclei were counted in at least ten randomly chosen microscopic fields to be able to recognize a potential effect of the cell culture media on cell numbers.

2.6. Measuring of growth factors by ELISA

Concentrations of VEGF-A and TNFα in supernatants of iBREC kept in ECGM or DMEM for one day, three or four days were measured with the Quantikine ELISA Canine VEGF-A Immunoassay Kit (CAVE00, biotechne; detects all splice variants of human, canine and bovine VEGF-A) or the Bovine TNFα ELISA Kit (ThermoFisher Scientific), respectively. Undiluted samples were processed in quadruplicates according to the manufacturers' instructions and analyte-dependent absorbance was measured at 450 nm (reference wavelength: 570 nm) 15 min after addition of the stop solution with an Infinite 200 Pro spectrophotometer controlled by Tecan i software (Tecan, Crailsheim, Germany). In parallel, standard curves (VEGF-A_{165}: 0–1250 pg/ml, TNFα: 0–4.8 ng/ml) were

Table 2. Primer pairs used for qRT-PCR[a].

Target and source sequence(s)	Primer sequence (5'→3')	Product size [bp]
ACTB NM_173979.3	$_{s}$ GCATTCACGAAACTACCTTCAAT $_{as}$ TGATCTTGATCTTCATTGTGCTG	171
HSP70 NM_005345.5	$_{s}$ GGAGGAGTTCAAGAGAAAACACA $_{as}$ GTAGAAGTCGATGCCCTCAAAC	157
VEGFA$_{188,\ 164,\ 120}$ NM_003376.5, NM_001287044.1, NM_001025370.2	$_{s}$ CCTGGTGGACATCTTCCAGGAGTA $_{as}$ CTCACCGCCTCGGCTTGTCACA	479; 407; 275

[a] s = sense primer; as = antisense primer.

generated, allowing accurate quantification of VEGF-A and TNFα in samples containing more than 20 pg/ml or 120 pg/ml, respectively. For measuring the concentrations of IL-6 in undiluted samples of the supernatant of iBREC cultivated in ECGM or DMEM for one to four days, the IL-6 Bovine Uncoated ELISA Kit (ThermoFisher Scientific) was used. Coating antibody (diluted 1:100 in 0.2 M sodium carbonate - bicarbonate buffer, pH 9.4 (BupH Carbonate/Bicarbonate buffer, 23832, ThermoFisher Scientific)) was placed in each well of a 96 well-plate (ELISA Plate High binding F, Sarstedt) and incubated overnight at room temperature in the dark. All following steps were carried out according to the manufacturer's instructions and analyte-dependent absorbance was measured at 450 nm (reference wavelength: 570 nm) 10 min after addition of the stop solution as described above. Accurate quantification of >75 pg/ml IL-6 was achieved with a standard curve (0–5000 pg/ml IL-6) performed in parallel.

2.7. Quantitative RT-PCR

Total RNA of confluent iBREC kept in the different culture media for one day, three or four days was extracted with the InviTrap Spin Universal RNA Mini Kit (Invitek, Berlin, Germany). The quality of the RNA was checked by agarose gel electrophoresis and by measuring the optical densities at 260 nm and 280 nm (A_{260} and A_{280}) with a NanoDrop 100 spectrophotometer (PeqLab, Erlangen, Germany); the A_{260}/A_{280} ratios of all RNA samples were between 1.95 and 2.05, confirming high quality. DNA was removed from the samples by DNase I treatment (Sigma) and cDNA was synthesized from ~0.5 µg of total RNA using the RevertAid H Minus First Strand cDNA Synthesis Kit (ThermoFisher Scientific). Quantitative RT-PCR (qRT-PCR) was carried out with the CFX Connect™Real-Time System (bio-rad). The PCR reaction mixture contained 1 µl of cDNA, two specific primers (0.2 µM each, listed in Table 2) and 5 µl of 2x qRT-PCR mix (iQ™ SYBR® Green Supermix, bio-rad) in a final volume of 10 µl. The PCR parameters were: Initial denaturation and enzyme activation at 95 °C for 3 min, 45 cycles of denaturation at 95 °C for 30 s, primer annealing at 58 °C for 20 s, and extension and quantification at 72 °C for 45 s. Each measurement included a melt curve analysis in the range 65 °C–95 °C with an increment of 0.5 K. Three splice variants VEGF-A_{188}, VEGF-A_{164} and VEGF-A_{120} were amplified in parallel with the primer pair listed in Table 2. As a housekeeping gene-encoded reference, the β-actin (ACTB) mRNA was quantified in parallel. qRT-PCR raw data were analyzed with the CFX Maestro™ Software (bio-rad) and relative changes in mRNA expression were calculated as $2^{-\Delta\Delta CT}$ with $\Delta CT = CT_{target} - CT_{ACTB}$, and $\Delta\Delta CT = \Delta CT_{DMEM} - \Delta CT_{ECGM}$. This method is based on a threshold set in the linear phase of amplification

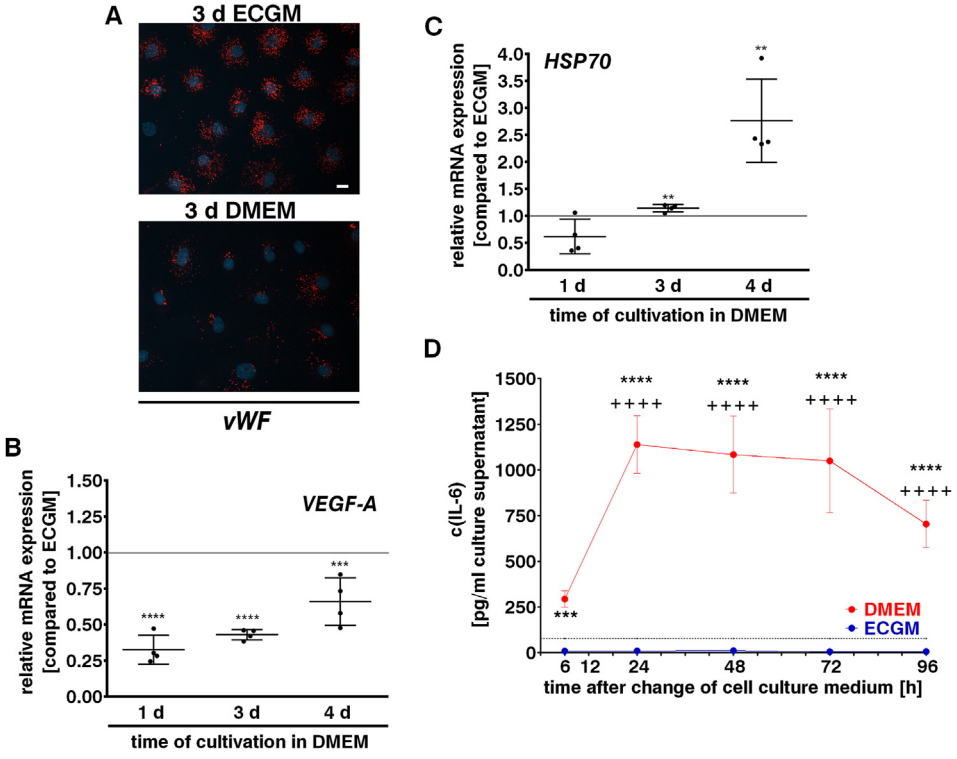

Figure 1. Cultivation in DMEM changed the EC-typic phenotype of iBREC and induced secretion of IL-6. (**A**) After having formed a stable confluent monolayer, iBREC were further cultivated in ECGM or DMEM for three days before cells were fixated for immunofluorescence staining. Expression of the EC-specific vWF was lower and more focused in the perinuclear region when cells had been kept in DMEM. Scale bar: 10 µm (**B, C**) iBREC were treated as described above and cells were harvested after one day, three or four days for preparation of mRNA and subsequent qRT-PCR analyses. Data were normalized and statistical analyses were performed as described in Material and Methods (see 2.7 and 2.8). Cultivation of the cells in DMEM changed the expression of **(B)** VEGF-A and **(C)** HSP70. **) $p < 0.01$, ***) $p < 0.001$, ****) $p < 0.0001$ compared to cells kept in ECGM for the same period; (**D**) Secretion of IL-6 into the cell culture supernatant of iBREC cultivated in ECGM or DMEM for six hours and one to four days was determined by ELISA. Cultivation in DMEM induced secretion of IL-6 starting as early as six hours after change of cell culture medium. The dotted line depicts the detection limit of 75 pg IL-6/ml. Statistical analyses were performed as described in Material and Methods (see 2.8). ***) $p < 0.001$ and ****) $p < 0.0001$ compared to cells kept in ECGM for the same period and $^{++++}$) $p < 0.0001$ compared to cells kept in DMEM for 6 h, N = 8 for all comparisons.

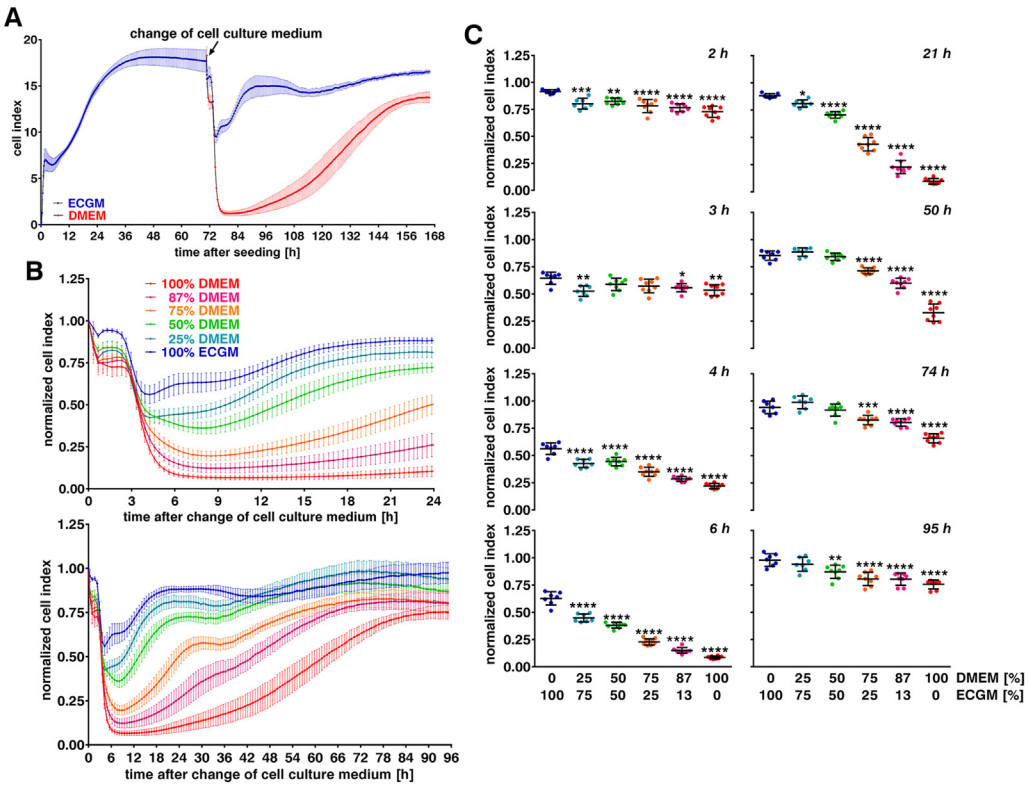

Figure 2. DMEM-cultivated iBREC showed a persistently decreased CI. iBREC were cultivated on gold electrodes in ECGM-MV for three days before the cell culture medium was completely replaced by ECGM, DMEM or mixtures thereof. CI values as measures of barrier function were determined continuously. (**A**) The CI steadily increased after seeding up to a plateau value reached two to three days later. Exchange of the cell culture medium for DMEM resulted in a dramatic and persistent decline of the CI whereas in ECGM cells showed only a transient effect. Mean and standard deviations of data obtained from at least six wells are shown. (**B, C**) After having formed a confluent monolayer, iBREC were further cultivated in mixtures consisting of DMEM and ECGM, and the CI was recorded. CI values - shown as means and standard deviations of data obtained from at least six wells - were normalized in relation to those measured immediately before the exchange of culture medium. The extent of the CI decline and its recovering depended on the fraction of DMEM in the blended media. Statistical analyses were performed at indicated time points as described in Material and Methods (see 2.8). *) $p < 0.05$, **) $p < 0.01$, ***) $p < 0.001$, ****) $p < 0.0001$ compared to 100% ECGM.

and CT defined as the cycle number at threshold signal intensity. PCR efficiency (E) was calculated according to equation $E = 10^{[-1/slope]}$; the investigated dilution range was between 0.05 ng and 50 ng cDNA. The PCR efficiencies with the used primer pairs were: *ACTB*, 1.94; *VEGFA*, 1.94; *HSP70*, 1.92.

2.8. Statistical analyses

To compare measured CI values at selected time points and values obtained from cell viability assays, one-way analyses of variance (ANOVA) followed by Tukey's test to determine differing groups were applied (Graph Pad Prism 6). For comparison of IL-6 concentrations measured by ELSA, a two-way ANOVA followed by Sidak's multiple comparisons test was used to detect differing groups (Graph Pad Prism 6). The non-parametric Wilcoxon signed rank test was applied to compare antigen-specific signals of Western blot analyses of extracts from cells kept in tested media to the hypothetical value of 1.0 of normalized signals from experiments with cells cultivated in ECGM (Graph Pad Prism 6). Student's t-test was used to analyze potential differences in transcript levels (SPSS Statistics 25, IBM, New York, USA). Differences resulting in p-values below 0.05 were considered significant. In addition to providing means and corresponding standard deviations, results were presented as scatter plots including this information.

3. Results and discussion

We investigated if cultivation of iBREC not in cell culture medium optimized for EC but in the commonly used DMEM affects their phenotype (e.g. expression of proteins typical or specific for microvascular EC), properties (e.g. forming a stable barrier), or even viability. The culture media compared, i.e ECGM and DMEM, or mixtures thereof, always contained 100 nM hydrocortisone and ECGS/H to ensure strong expression of TJ-proteins by the cells [4, 24, 31].

3.1. Cultivation of iBREC in DMEM affected subcellular localization of vWF, mRNA expression of VEGF-A and HSP70, and induced secretion of IL-6

Expression of the vWF, a subunit of factor VIII exclusively located in so-called Weibel-Palade bodies, is considered a specific characteristic of EC cells [22, 23]. Therefore, we investigated whether prolonged incubation of iBREC in DMEM affected its expression or subcellular localization (Figure 1A). After a confluent iBREC monolayer had been formed, cell culture medium ECGM-MV was replaced by ECGM or DMEM and three days later cells were fixated for immunofluorescence stainings (Figure 1A). These showed not only a lowered expression of vWF, but that it was less evenly distributed in the cytoplasm (with strong signals only close to the perinuclear region) after DMEM-cultivation, suggesting a less endothelial cell-like phenotype.

Inspired by the puzzling observation that VEGF-A proteins were not produced by primary or immortalized bovine REC (cultivated in DMEM or ECGM) despite the corresponding mRNA being expressed, we assessed VEGF-A-specific mRNA and the potential secretion of the encoded protein after cultivation of confluent iBREC in DMEM or ECGM [6, 15, 16]. VEGF-A-mRNA was indeed expressed over the entire period of four days, but after one day of cultivation it was considerably lower when cells were kept in DMEM. The amount increased over time without reaching the levels expressed by cells cultivated in ECGM (Figure 1B). However, the amounts of VEGF-A secreted into the culture medium were below the detection limit of the ELISA (≤20 pg/ml) under all conditions investigated. This confirms that confluent iBREC do not express or secrete relevant amounts of VEGF-A protein(s) [6, 16]. Of course, it cannot be completely ruled out that some VEGF-A is stored in intracellular vesicles,

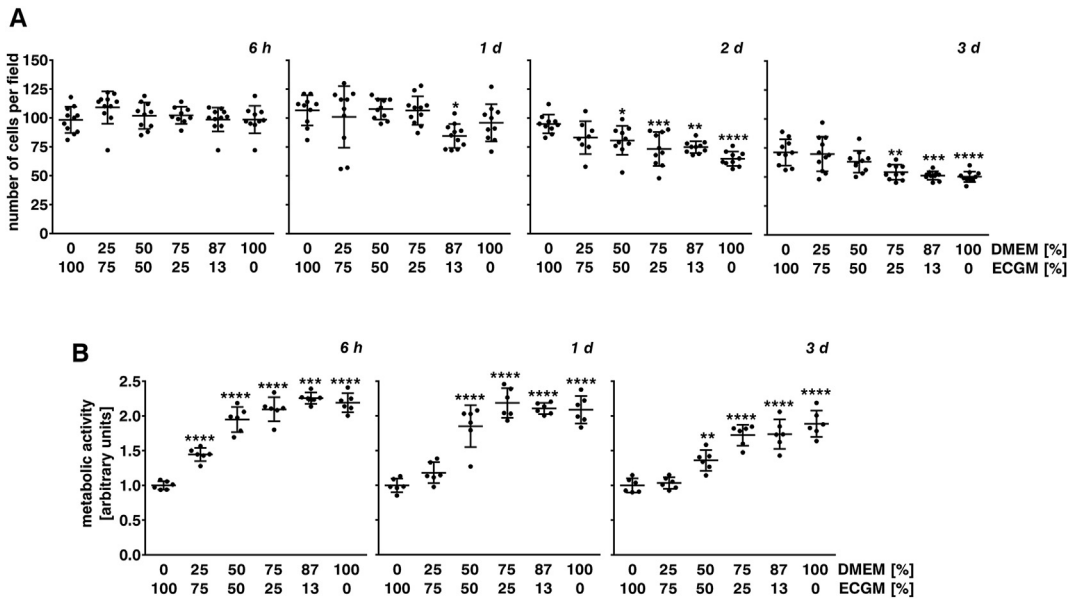

Figure 3. DMEM decreased cell density in the monolayer but enhanced metabolic activity of iBREC. (**A**) Confluent iBREC cultivated in ECGM, DMEM or mixtures thereof were fixated, and DAPI-stained nuclei were counted in at least ten randomly chosen microscopic fields. In cell culture media consisting mainly of DMEM, significant numbers of cells were lost after two or three days. (**B**) Measuring the enzymatic activity of NAD(P)H-dependent oxidoreductases of iBREC cultivated in the different cell culture media for up to three days revealed that DMEM or mixtures consisting of at least 50% DMEM considerably increased the basic metabolic activity of iBREC. Statistical analyses were performed as described in Material and Methods (see 2.8). *) $p < 0.05$, **) $p < 0.01$, ***) $p < 0.001$, ****) $p < 0.0001$ compared to 100% ECGM.

but a significant contribution of an amount below the detection limits of Western Blot analyses or ELISA is unlikely.

We also assessed HSP70, because its increased expression is considered a general stress response of cells: Indeed, amounts of HSP70-mRNA in iBREC strongly and significantly increased during cultivation of the cells in DMEM (Figure 1C) [27]. As dermal microvascular endothelial cells secrete IL-6 as response to stress, we also investigated whether iBREC express such a behavior during cultivation in DMEM (Figure 1D) [28]. Indeed, IL-6 was then detected in the supernatant of these cells whereas the concentration of this cytokine was below the detection limit for iBREC cultivated in ECGM. Secretion of IL-6 appeared as early as six hours after changing the cell culture medium to DMEM and was stable for at least four days.

All of these results clearly showed that iBREC quickly and persistently change their EC-typic phenotype accompanied by cellular stress responses when cultivated in DMEM.

3.2. Cultivation of iBREC in DMEM resulted in an immediate and persistent decline of the cell index

Reflecting their most important in vivo function, monolayers of cultivated REC form a stable barrier [3, 31]. To assess their barrier stability, we performed continuous and sensitive measurements of the cell index (CI) of iBREC cultivated on gold electrodes [6, 29]. Because the cells remain in the incubator during the entire period of measurements, determination of the CI is less prone to disturbances caused by variations of environmental conditions. This allows even subtle or transient changes due to impaired paracellular and transcellular flow to be easily detected [6, 29, 30, 32]. Because the cells directly attach to the electrodes, the nature of their adhesion co-determines the measured CI values [30, 33].

After seeding the cells in ECGM-MV the CI continuously increased and reached a stable and high CI about three days later ($CI = 17.63 \pm 1.114$; $n = 14$) indicative of a confluent monolayer of iBREC forming a stable barrier typical of microvascular REC (Figure 2A). As previously observed, replacing the cell culture medium ECGM-MV by ECGM resulted in a transient decline of the CI ($CI = 9.71 \pm 0.60$, 4 h after exchange; $n = 6$) coinciding with the appearance of small intercellular gaps as revealed by immunofluorescence staining with antibodies specific for the TJ-protein claudin-5 [16]. The decline which cannot be prevented by inhibiting VEGF-A, is caused by components of FBS not yet identified [16]. Within hours the CI recovered and remained stable for at least four days accompanied by strong expressions of TJ-proteins claudin-1, claudin-5 and AJ-protein VEcadherin (e.g. $CI = 15.29 \pm 1.05$ and $CI = 16.71 \pm 0.15$, 21 h or 95 h after exchange, respectively; $n = 6$; Figure 2A, Figure 4). In contrast, cultivation in DMEM resulted in a prompt and dramatic drop of the CI (e.g. $CI = 1.58 \pm 0.24$, 6 h after exchange; $n = 8$), which only very slowly increased again and remained below normal values even four days later ($CI = 13.87 \pm 0.6$; $n = 8$, Figure 2A). This effect was even more pronounced when supplement-free DMEM or that supplemented only with hydrocortisone was used (data not shown). It is of interest, that supplementation of DMEM with ECGS/H and hydrocortisone is not sufficient to stabilize the then low CI although both components have been shown to maintain the phenotype of microvascular EC [19, 31]. In mixtures consisting of ECGM and DMEM, the extent of iBREC's CI decline and its recovery were dependent on the portion of DMEM, mixtures with ≥50% DMEM resulted in the strongest response (Figure 2B, C).

All the substantial culture medium-dependent effects revealed by CI measurements, which were even stronger than those observed when cells were exposed to the permeability-inducing VEGF-A_{165} applied at a concentration of 1 nM, clearly demonstrate that essential properties of REC are dramatically affected by short-term or prolonged cultivation in suboptimal DMEM [6, 16].

3.3. Monolayer cell density and metabolic activity of iBREC were affected by cultivation in DMEM

The observed decline of the CI might be caused by structural rearrangement of the monolayer associated with loss of cells. To test this hypothesis, confluent monolayers of iBREC were kept in DMEM/ECGM mixtures for up to 72 h and numbers of nuclei of fixated cells were counted at selected time points. After cultivation for three days in cell culture medium consisting of at least 75% DMEM, slightly but significantly lower cell numbers were measured (Figure 3A). Interestingly, cells

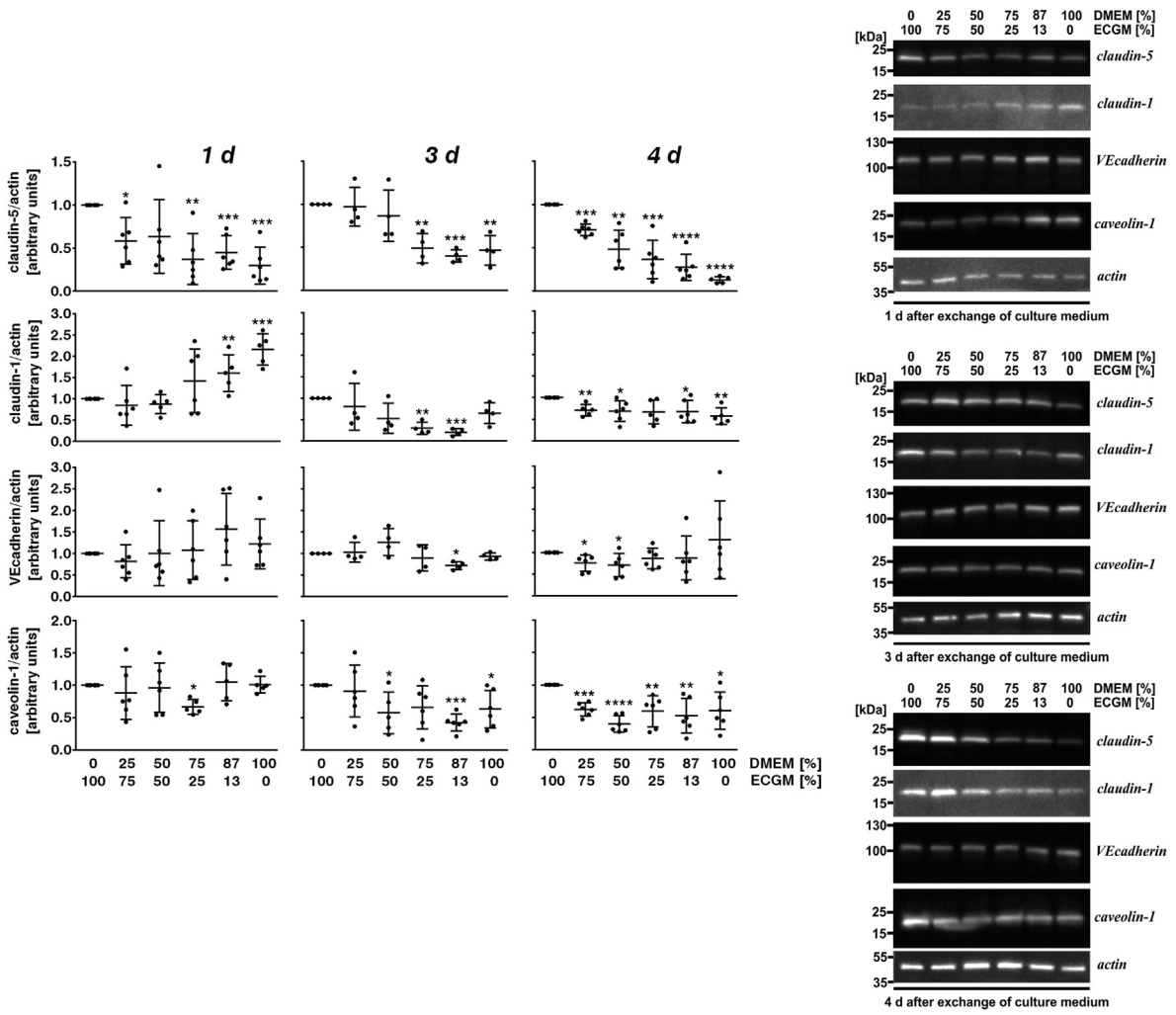

Figure 4. DMEM decreased expression of proteins involved in the regulation of paracellular and transcellular flow. Confluent iBREC were cultivated in ECGM, DMEM or mixtures thereof for one, three or four days, before cells were harvested for preparation of cell extracts to be analyzed by Western blot. Expressions of claudin-5 and claudin-1 were lower after longer cultivation in all cell culture media consisting at least partly of DMEM. More claudin-1 was measured after one day, whereas the amount of claudin-5 then started to decline and was barely detectable after incubation in 75%–100% DMEM for four days. Expression of caveolin-1 was significantly lower only after prolonged cultivation in all media consisting at least partly of DMEM. In contrast, expression of VEcadherin was similar under all conditions. Signals were normalized and statistical analyses were performed as described in Material and Methods (see 2.4 and 2.8).*) $p < 0.05$, **) $p < 0.01$, ***) $p < 0.001$, ****) $p < 0.0001$ compared to 100% ECGM. Each dot represents the result of one western blot experiment of which representative images are shown. For original images, please refer to supplementary Figure 1 (one day of cultivation), supplementary Figure 2 (three days of cultivation) or supplementary Figure 3 (four days of cultivation), respectively.

cultivated in DMEM or DMEM-dominated mixtures showed strongly increased metabolic activity, a highly significant effect already evident after several hours (Figure 3B). It can be speculated that iBREC up-regulate their metabolic activity as a first step to compensate lost cells by proliferation and reestablish the integrity of the monolayer.

3.4. Cultivating iBREC in DMEM changed expression or subcellular localization of proteins involved in the regulation of paracellular flow or transcellular transport

An increased paracellular flow due to disruption or changed compositions of TJ or AJ could also have caused the observed decline of the CI values or at least contributed to this striking effect [6, 32]. Presence of the TJ proteins claudin-1 and claudin-5 is associated with a strong barrier formed by a REC monolayer which is reflected by their high trans-endothelial electric resistance or CI values [4, 6, 12, 24]. Expression of these proteins was assessed by Western blot analyses of cell extracts prepared from cells exposed to the different cell culture media for one, three or four days (Figure 4). Cells kept for four days in media containing DMEM expressed significantly less caudin-5 which was barely detectable after cultivation in pure DMEM. Its expression had already started to decline significantly after one day in ≥75% DMEM (Figure 4, upper panel). It is of interest in the context of cell type authenticity that expression of claudin-5 is specific for EC and strong particularly by those originating from microvessels [34, 35]. In contrast to claudin-5, expression of claudin-1 was not changed by cultivating iBREC in ≤75% DMEM for only one day, but lower amounts of this protein were measured after four days in all media containing DMEM (Figure 4, second panel from the top). Interestingly, iBREC kept in ≥75% DMEM for one day expressed significantly more claudin-1. As the CI values were still low then, the surplus amount of claudin-1 was obviously not sufficient to compensate the early loss of TJ-protein claudin-5 and preserve barrier integrity (Figure 2B, C). Reduced levels of claudin-1 - a TJ protein expressed by endothelial and epithelial cells - are associated with the VEGF-A_{165}-induced higher permeability of barriers formed by retinal EC from different species when applied at concentrations of 1 nM [4, 5, 6, 7, 12, 16]. It is surprising that when loss of both TJ-proteins claudin-1 and claudin-5 was most substantial after extended cultivation in DMEM-containing media,

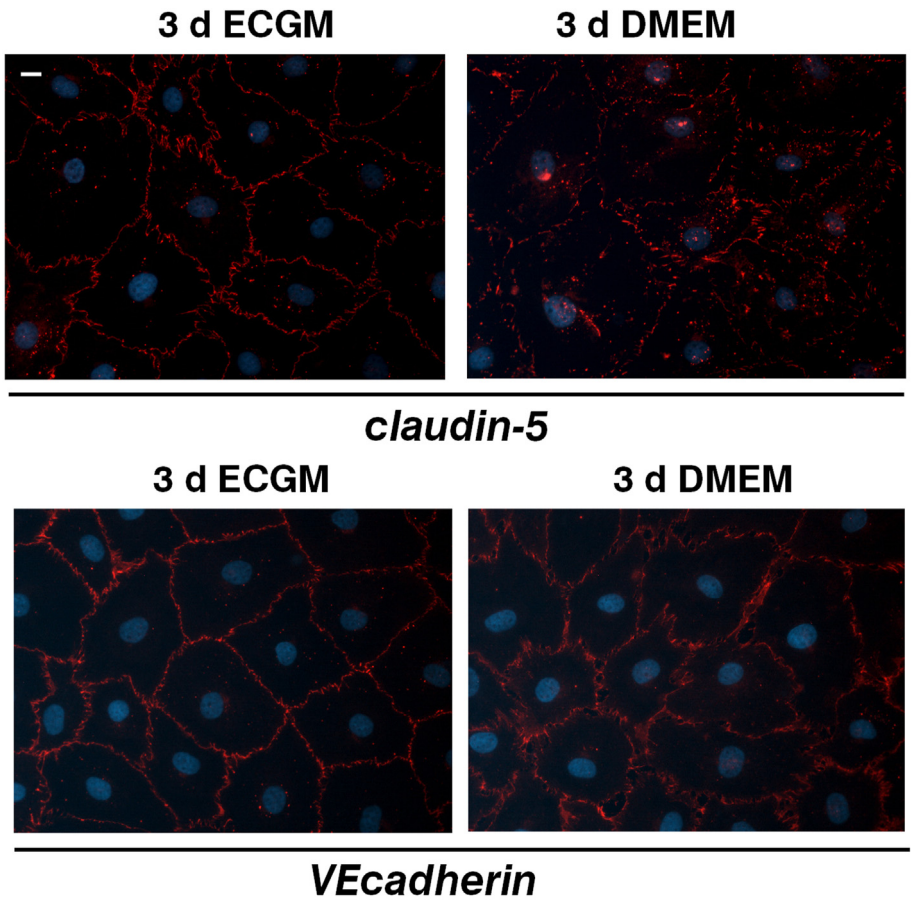

Figure 5. DMEM changed subcellular localization of claudin-5 and VEcadherin. After having cultivated iBREC in ECGM or DMEM for three days, cells were fixated for immunofluorescence stainings with antibodies specific for claudin-5 or VEcadherin, respectively. With both antibodies, the pronounced and homogenous staining of the plasma membrane appeared either disrupted or more diffuse after cultivation in DMEM. Scale bar: 10 μm.

the CI values had already started to recover. However, these values were still far from normal and in the range of those observed after prolonged treatment with the permeability-inducing growth factor VEGF-A_{165} [6, 16]. In contrast to the investigated TJ proteins, expression of the EC-specific VEcadherin - also involved in the regulation of paracellular flow – was similar by cells cultivated in the different media and over the entire period investigated (Figure 4, third panel from the top). We also assessed possible DMEM effects on the subcellular localizations of TJ-protein claudin-5 and AJ-protein VEcadherin in iBREC, because their relocation from the plasma membrane was found to be associated with an increased permeability of (retinal) EC [25, 26, 36, 37]. Indeed, after having cultivated the cells with DMEM for three days, a weaker and often interrupted claudin-5-specific staining as well as a more diffuse staining of VEcadherin at the plasma membranes were observed (Figure 5).

Taken together, these results indicate that the still lowered CI after keeping iBREC in pure or even diluted DMEM for several days very likely results from an increased paracellular flow due to functionally impaired TJ and AJ. These changes are obviously different from those caused by VEGF-A_{165} because only expression of claudin-1, but not of claudin-5, was lowered by prolonged exposure of iBREC to the growth factor [4, 5, 6, 16]. Short-term treatment with 1 nM VEGF-A_{165} for a few hours did not change the expressed amounts of the two TJ-proteins or of AJ-protein VEcadherin at all and led to an only subtle and transient relocation of the latter [6, 7]. These findings rule out a VEGF-dependent autocrine effect as mechanism of barrier impairment and, accordingly, expression of VEGF-A (mRNA or protein) was not induced by cultivation of iBREC in DMEM (Figure 1B). Tumor necrosis factor (TNF)α-induced permeability is associated with depletion of claudin-5 from the plasma membrane, observed already after few hours of exposure to this cytokine [36, 37]. Based on this observation and a claudin-5 staining pattern similar to that seen after treatment with TNFα, we also tested if this cytokine might contribute to the DMEM-caused effects, but it was not detectable over the entire period of cultivation.

We included caveolin-1 in the analyses of candidate proteins expressed by EC and potentially affected by cultivation in DMEM, because the major component of caveolae is not only involved in the regulation of transcellular transport and intracellular signaling, but its lowered expression is also associated with phenotypic conversion in the course of cell differentiation: Expression of caveolin-1 was indeed significantly lower after prolonged cultivation of the cells in DMEM or DMEM-containing mixtures of media (Figure 4, bottom panel) [39, 40].

Our study reveals that cultivation of iBREC in standard DMEM dramatically changed their essential properties resulting in at least partial loss of their typical features. These observations provide a straightforward explanation for the apparently inconsistent behavior of REC reported by several research groups which used in their experiments different cell culture media, i.e. DMEM, not meeting the special requirements of microvascular EC. As the supplements shown to maintain the phenotype of microvascular EC, i.e. hydrocortisone and ECGS/H, were part of the cell culture media investigated, iBREC's differing responses likely result from the different compositions of the basal cell culture media, namely Dulbecco's modified Eagle's medium or ECGM-MV [19, 20, 31]. Unfortunately, the exact composition of ECGM-MV is not known for propriety reasons, but it is a reasonable speculation that its composition is based on that of MCDB131, a cell culture medium composed to meet the special requirements of microvascular EC [19]. One striking difference is MCDB131's ten times higher concentration of Mg^{2+} (i.e. 10 mM) compared to DMEM, and Knedler and Ham indeed

observed a dramatic rise of the growth response of human dermal microvascular EC at high concentrations of Mg^{2+} [19]. This cation is an important component of integrins and their complexes, e.g. the fibronectin receptor, and one might therefore speculate that its high concentration could improve adhesion of the microvascular EC to the extracellular matrix protein fibronectin [41]. A stronger adhesion of the cells might be reflected by a higher CI due to their direct interaction with the gold electrodes, and this was indeed observed for iBREC cultivated in ECGM but not in DMEM (Figure 2) [30, 33]. However, stabilization of EC-typic characteristics of iBREC or any microvascular EC-type by ECGM is very likely based on the combination of various components of the cell culture medium and not one specified ingredient, but unfortunately this detailed information is not accessible. Although the experiments of this study were performed with immortalized REC, similar behavior of primary REC can be reasonably assumed, because bovine or human primary REC and iBREC respond to stimulation by various (human) growth factors in the very same ways [3, 4, 9, 10, 11, 12, 31]. Our results also underline that assessing a single parameter, such as the metabolic activity of cells, is not sufficient to confirm vitality and authenticity of cells, and to rule out detrimental effects of environmental factors. Therefore, additional phenotypic characterization of cells, e.g. by confirming expression and subcellular localizations of validated marker proteins, is imperative to ensure the relevance of in vitro models.

In basic and pharmacological research, reliable cell culture models can be a valuable alternative to the time-consuming and expensive animal models, helping to limit their use in accordance with ethical considerations. Particularly promising approaches to better understand the pathogeneses of the sight-threatening macular edema secondary to diabetic retinopathy or retinal vein occlusion are based on co-culture models with retinal endothelial cells, retinal pericytes and Muller cells mimicking the neurovascular unit. Development of such models, highly useful to find and test novel therapeutics, crucially depends on careful adjustment of conditions to conserve the specific properties of the different cell types.

4. Conclusion

Cultivation of retinal endothelial cells - and very likely any kind of endothelial cells - in cell culture media optimized for these cell types is not only more appropriate but even crucial to obtain relevant results. This has to be taken into consideration when in vitro studies with (retinal) endothelial cells are planned or their outcomes evaluated.

Declarations

Author contribution statement

Catharina Busch, Margrit Hollborn, Heidrun L. Deissler: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Matus Rehak: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Gabriele E. Lang, Gerhard K. Lang, Peter Wiedemann, Armin Wolf: Analyzed and interpreted the data; Wrote the paper.

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Declaration of interests statement

The authors declare no conflict of interest.

Additional information

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