Research article

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

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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 (PIGF) 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...
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), 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% CO₂, the cells went through passing 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 (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% CO₂, the cells went through passing 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].

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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=(Zi-Z0)/15 Ω (RTCA Software 2.0, Acea). In this formula, Zi is the impedance measured at an individual time point and Z0 the impedance read at the start of the experiment [29]. Cells were cultivated in wells of an E-Plate 16 PET (0.5 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 (~2 × 10⁴ cells per fibronectin-coated well) were seeded in 200 μl ECGM-MV and cultivated...
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 × 10⁴ 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 cm², Sarstedt, Nuembrecht, Germany) were further cultivated in ECGM 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 (biotecne)/10⁶ 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 after agitation, the suspensions were centrifuged (30 min, 21200 x g, 4 °C) and the protein concentrations of the supernatants were determined using 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 (Roht, 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 dilution/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 (CVE900, 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, 0–1250 pg/ml, TNFα: 0–4.8 ng/ml) were measured at 540 nm, 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.

The results were converted to graphs showing means and standard deviations using Graph Pad Prism 6 (Graph Pad, San Diego, USA).
Quantitative RT-PCR
was checked by agarose gel electrophoresis and by measuring the optical
versal RNA Mini Kit (Invitek, Berlin, Germany). The quality of the RNA
VEGFA188, 164, 120
stop solution as described above. Accurate quanti-
ation of VEGF-A and TNF-α
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 Con-
nect™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 45 s, and extension and quanti-
fication 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-A188, VEGF-A164 and VEGF-A120 were amplified in parallel
with the primer pair listed in Table 2. As a housekeeping gene-encoded
β-actin (ACTB) mRNA was quanti-
ed in parallel. C. Busch et al. Heliyon 7 (2021) e06037

2.7. Quantitative RT-PCR

Total RNA of confluent iBREC kept in the different culture media for
day one, three or four days was extracted with the Invitrap Spin Uni-
versal 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.

Table 2. Primer pairs used for qRT-PCR.

| Target and source sequence(s) | Primer sequence (5′→3′) | Product size [bp] |
|-----------------------------|-------------------------|------------------|
| ACTB NM_173979.3             | a TGATGTCGATCTCATGTTGCTG | 171              |
| HSP70 NM_005345.5            | a GGAGGATCTCGAGGAACACCA | 157              |
| VEGF-A188, 164, 120          | a GCCATTCAAGAATACCTTCAAT | 479; 407; 275    |
|                             | a CTTGGTTCATCCACGAGGACGTA |                   |
|                             | a CCCTGGTGGACATCTCCAGGAGTA |                   |
|                             | a GCATTCACGAACTACCTTCAAT |                   |

* s = sense primer; as = antisense primer.

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
fixed for immunofluorescence staining. Expres-
sion 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 statisti-
cal analyses were performed as described in
Material and Methods (see 2.7 and 2.8). Cultiva-
tion of the cells in DMEM changed the expres-
sion 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
 cultivating in ECGM or DMEM for six hours and
one to four days was determined by ELISA. Cultiva-
tion 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 ana-
lyses 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.
and CT defined as the cycle number at threshold signal intensity. PCR efficiency (E) was calculated according to equation $E = 10^{(-1/\text{slope})}$; 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, 1.92; 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 measured CI values at selected time points and values obtained from cell viability assays, one-way analyses of variance (ANOVA) followed by Sidak’s multiple comparisons test were used 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. Mean 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.

### 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].

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 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, but the transition of mRNA to secreted VEGF-A could not be detected over a period of four days.
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 a 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-typical 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, 16]. 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 ¼ 17.63 ± 1.114; n ¼ 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 ¼ 9.71 ± 0.60, 4 h after exchange; n ¼ 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 ¼ 15.29 ± 1.05 and CI ¼ 16.71 ± 0.15, 21 h or 95 h after exchange, respectively; n ¼ 6; Figure 2A, Figure 4). In contrast, cultivation in DMEM resulted in a prompt and dramatic drop of the CI (e.g. CI ¼ 1.58 ± 0.24, 6 h after exchange; n ¼ 8), which only very slowly increased again and remained below normal values even four days later (CI ¼ 13.87 ± 0.6; n ¼ 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-A165 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 fixed 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...
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 upregulate 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 and 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 claudin-5 which was barely detectable after cultivation in pure DMEM. Its expression had already started to decline significantly after one day in 75%–100% 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%–100% 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-A165-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,
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-A165 [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-A165 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-A165 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, iBRECs 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 Mg²⁺ (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 Mg2+ [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: Concepted and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Matus Rehak: Concepted 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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Data included in article supplementary material/referenced in article.

Declaration of interests statement

The authors declare no conflict of interest.

Additional information

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References

[1] H. Ozaki, A.Y. Yu, N. Della, K. Ozaki, D.J. Luna, H. Yamada, S.F. Hackett, N. Okamoto, D.F. Zack, G.L. Semenz, P.A. Campochiaro, Hypoxia inducible factor-1 alpha is increased in ischemic retinal: temporal and spatial correlation with VEGF expression, Invest. Ophthalmol. Vis. Sci. 40 (1999) 182–189.
[2] T. Qian, Q. Xu, A.M. Jousen, M.W. Clemens, W. Qin, K. Miyamoto, H. Hassessian, S.J. Wiegand, J. Rudge, G.D. Vancopoulous, A.P. Adams, VEGF-initiated blood-retinal barrier breakdown in early diabetes, Invest. Ophthalmol. Vis. Sci. 42 (2001) 2408–2413.
[3] D.A. Antonetti, A.J. Barber, L.A. Hollinger, E.B. Woltpert, T.W. Gardner, Vascular endothelial growth factor induces rapid phosphorylation of tight junction proteins occludin and zonula occludens-1, J. Biol. Chem. 274 (1999) 23453–23467.
[4] H. Deissler, H. Deissler, G.E. Lang, Inhibition of protein kinase C is not sufficient to prevent or reverse effects of VEGF on claudin-1 and permeability in microvascular retinal endothelial cells, Invest. Ophthalmol. Vis. Sci. 51 (2010) 525–532.
[5] H.L. Deissler, H. Deissler, G.K. Lang, G.E. Lang, VEGF but not PIGF disturbs the barrier of retinal endothelial cells, Exp. Eye Res. 115 (2013) 162–171.
[6] H.L. Deissler, G.K. Lang, G.E. Lang, Inhibition of single routes of intracellular signaling is not sufficient to neutralize the biophysical disturbance of a retinal endothelial cell barrier induced by VEGF-165, Cell. Physiol. Biochem. 42 (2017) 1493–1513.
[7] H.L. Deissler, J.N. Stutzer, G.K. Lang, S. Grisanti, G.E. Lang, M. Ranjbar, VEGF receptor 2 inhibitor nintedanib completely reverses VEGF-165-induced disturbances of barriers formed by retinal endothelial cells or long-term cultivated ARPE-19 cells, Exp. Eye Res. 194 (2020) 108004.
[8] H. Deissler, H. Deissler, G.K. Lang, G.E. Lang, Generation and characterization of iBREC: novel hTERT-immortalized bovine retinal endothelial cells, Int. J. Mol. Med. 15 (2005) 65–70.
[9] H.L. Deissler, H. Deissler, G.K. Lang, G.E. Lang, Ranibizumab efficiently blocks migration but not proliferation induced by growth factor combinations including VEGF in retinal endothelial cells, Graefes Arch. Clin. Exp. Ophthalmol. 251 (2013) 2345–2353.
[10] R. Castellon, H.K. Hamdi, I. Sacerio, A.M. Aoki, M.C. Kenney, A.V. Ljubimov, Effects of angiogenic growth factor combinations on retinal endothelial cells, Exp. Eye Res. 74 (2002) 525–535.
[11] E.A. Stewart, G.J. Samaranayake, A.C. Browning, A. Hopkinson, W.M. Amoako, Comparison of choroidal and retinal endothelial cells: characteristics and response to VEGF-inosine and anti-VEGF treatment, Exp. Eye Res. 93 (2011) 761–766.
[12] S. Suarez, G.W. McCollum, C.A. Bertz, B. Yang, M.E. Capozzi, J.S. Penn, Modulation of VEGF-induced retinal vascular permeability by peroxisome proliferator-activated receptor-β/δ, Invest. Ophthalmol. Vis. Sci. 55 (2014) 8232–8240.
[13] A.C. Browning, E.P. Halligan, E.A. Stewart, D.C. Swan, R. Dove, G.F. Samaranayake, W.M. Amoako, Comparative gene expression profiling of human umbilical vein endothelial cells and ocular vascular endothelial cells, Br. J. Ophthalmol. 96 (2012) 128–132.
[14] H. Huang, A. Lennikov, M.S. Saddala, D. Guzel, D.J. Grab, A. Khalyfa, L. Fan, Placental growth factor negatively regulates retinal endothelial cell barrier function through suppression of glucose-6-phosphate dehydrogenase and antioxidant defense systems, Faseb. J. 33 (2019) 13695–13709.
[15] V. Simonne-Pinatel, M. Guerin, P. Chollet, M. Peray, S. Blamires, F. Malecaze, J. Plouet, Vasculotropin-VEGF stimulates retinal capillary endothelial cells through an autocrine pathway, Invest. Ophthalmol. Vis. Sci. 39 (1998) 3393–3400.
[16] H.L. Deissler, K. Sommer, G.K. Lang, G.E. Lang, Transport and fate of a fluorescent VEGF-A165-challenged retinal endothelial cells, Exp. Eye Res. 198 (2020) 108156.
[17] E.A. Stewart, S. Saker, W.M. Amoako, Desametasone reverses the effects of high glucose on human retinal endothelial cell permeability and proliferation in vitro, Exp. Eye Res. 151 (2016) 75–81.
[18] C.F. Wang, J.R. Yuan, D. Qin, J.F. Gu, B.J. Zhao, L. Zhang, D. Zhao, J. Chen, X.P. Hou, N. Yang, W.Q. Bu, J. Wang, C. Li, G. Tian, Z.B. Dong, L. Feng, X.B. Jia, Protection of tauroursodesoxycholic acid on high-glucose-induced human retinal microvascular endothelial cell dysfunction and streptozotocin-induced diabetic retinopathy rats, J. Ethnopharmacol. 185 (2016) 162–175.
A. Knedler, R.G. Ham, Optimized medium for clonal growth of human microvascular endothelial cells with minimal serum. In Vitro Cell, Dev. Biol. 23 (1987) 481–491.

R. Dulbecco, G. Freeman, Plaque production by the polyoma virus, Virology 8 (1959) 396–397.

T.J. McKee, S.V. Komarova, Is it time to reinvent basic cell culture medium? Am. J. Physiol. Cell Physiol. 312 (2017) C624–C626.

E.A. Jaffe, Endothelial cells and the biology of factor VIII, N. Engl. J. Med. 296 (1977) 477–483.

E.A. Jaffe, Synthesis of factor VIII by endothelial cells, Ann. N. Y. Acad. Sci. 401 (1982) 163–170.

E.A. Jaffe, Endothelial cells and the biology of factor VIII. In Vitro Cell, Dev. Biol. 23 (1987) 481–491.

R. Dulbecco, G. Freeman, Plaque production by the polyoma virus, Virology 8 (1959) 396–397.

E.A. Jaffe, Endothelial cells and the biology of factor VIII, N. Engl. J. Med. 296 (1977) 477–483.

E.A. Jaffe, Synthesis of factor VIII by endothelial cells, Ann. N. Y. Acad. Sci. 401 (1982) 163–170.

E.A. Felinski, A.E. Cox, B.E. Phillips, D.A. Antonetti, Glucocorticoids induce transactivation of tight junction genes occludin and claudin-5 in retinal endothelial cells via a novel cis-element, Exp. Eye Res. 86 (2008) 867–878.

E. Dejana, E. Tournier-Lasserve, B.M. Weinstein, The control of vascular integrity by endothelial cell junctions: molecular basis and pathological implications, Dev. Cell 16 (2009) 209–221.

J.H. Jeong, H.K. Nguyen, J.E. Lee, W. Suh, Therapeutic effect of apatinib-loaded nanoparticles on diabetes-induced retinal vascular leakage, Int. J. Nanomed. 11 (2016) 3101–3109.

L. Jornot, M.E. Mirault, A.F. Junod, Differential expression of hsp 70 stress proteins in human endothelial cells exposed to heat shock and hydrogen peroxide, Am. J. Respir. Cell Mol. Biol. 5 (1991) 265–275.

L.L. Strohl, J.B. Zang, W. Ding, M. Manni, X.K. Zhou, L. DeMaio, N.S. Harhaj, R.C. Scaduto Jr., Hydrocortisone decreases retinal endothelial cell water and solute flux coincident with increased content and decreased phosphorylation of occludin, J. Neurochem. 80 (2000) 667–677.

I. Bischoff, M.C. Horbinger, B.A. Mayer, A. Beyerle, J. Wegener, R. Fürst, Pitfalls in assessing microvascular endothelial barrier function: impedance-based devices versus the classic macromolecular tracer assay, Sci. Rep. 6 (2016) 25671.

J.M. Atenza, J. Zhu, X. Wang, X. Xu, Y. Abassi, Dynamic monitoring of cell adhesion and spreading on microelectronic sensor arrays, J. Biomol. Screen 10 (2005) 795–805.

K. Morita, H. Sanaki, M. Furuse, S. Tsukita, Endothelial claudin: claudin-5/TMVCF constitutes tight junction strands in endothelial cells, J. Cell Biol. 147 (1999) 185–194.

M.S. Kluger, P.R. Clark, G. Tellides, V. Gerke, J.S. Pober, Claudin-5 controls intercellular barriers of human dermal microvascular but not human umbilical vein endothelial cells, Arterioscler. Thromb. Vasc. Biol. 33 (2014) 489–500.

C.A. Avileña, C.M. Lin, S.F. Abcouwer, A.F. Ambrósio, D.A. Antonetti, TNF-α signals through PKCζ/NF-κB to alter the tight junction complex and increase retinal endothelial cell permeability, Diabetes 59 (2010) 2782–2782.

A. Gonçalves, L. Almeida, A.P. Silva, C. Fontes-Ribeiro, A.F. Ambrósio, A. Cristóvão, R. Fernandes, The dipeptidyl peptidase-4 (DPP-4) inhibitor sitagliptin ameliorates retinal endothelial cell dysfunction triggered by inflammation, Biochem. Pharmacol. 102 (2018) 833–838.

H.N. Fridolfsson, D.M. Roth, P.A. Insel, H.H. Patel, Regulation of intracellular signaling and function by caveolin, Faseb. J. 28 (2014) 3823–3831.

X.M. Wang, Y. Zhang, H.P. Kim, Z. Zhou, C.A. Peghali-Bostwick, F. Liu, E. Ibedigbe, X. Xu, T.D. Oury, N. Kaminski, A.M. Choi, Caveolin-1: a critical regulator of lung fibrosis in idiopathic pulmonary fibrosis, J. Exp. Med. 203 (2006) 2895–2906.

S.R. Akiyama, Integrins in cell adhesion and signaling, Hum. Cell 9 (1996) 181–186.