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Thekla Jaeckel, Lilla Knels, Monika Valtink, Richard Hw Funk, Katrin Engelmann. Serum-free SFM corneal organ culture medium but not conventional MEM organ culture medium protects human corneal endothelial cells from apoptotic and necrotic cell death. *British Journal of Ophthalmology*, 2010, 95 (1), pp.123. 10.1136/bjo.2010.183418 . hal-00588493

HAL Id: hal-00588493

<https://hal.science/hal-00588493>

Submitted on 24 Apr 2011

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Serum-free SFM corneal organ culture medium but not conventional MEM organ culture medium protects human corneal endothelial cells from apoptotic and necrotic cell death

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key words: human corneal endothelium, serum-free medium, protection, apoptosis, corneal organ cultivation medium

word count: 3096

ABSTRACT

Aim: To evaluate the influence of organ culture media on corneal endothelial cell survival.

Methods: The human corneal endothelial cell line HCEC-12 was cultured in five different media: HCEC growth medium F99_{HCEC}, standard corneal organ culture medium MEM+2% FCS, MEM+5% FCS, and humanised, serum-free medium Endothelial-SFM (with and without antibiotics). Part of the cells were treated with 0.5µmol/l staurosporine and examined for signs of apoptosis by assessing mitochondrial membrane polarisation state (intravital JC-1 staining), YO-PRO-1 and propidium iodide staining, determining fragmentation of nuclei by sub-G1 DNA content, immunocytochemistry for cleaved caspase-3, cleaved caspase-8, Bax, Bcl-2, and western blotting for cleaved caspase-3 and cleaved PARP.

Results: The number of apoptotic cells in untreated control cultures was significantly higher in MEM compared to F99_{HCEC} and SFM. Staurosporine treatment induced apoptosis in all tested cultures to varying degrees. Cells cultured in MEM showed stronger staining for cleaved caspase-3, cleaved caspase-8, Bax, Bcl-2, and cleaved PARP, increased sub-G1 DNA content, more propidium iodide and YO-PRO-1 positive cells, and more mitochondria with depolarised membranes. All parameters were significantly higher in MEM compared to F99_{HCEC} and SFM. SFM cultures were significantly less susceptible to cell stress.

Conclusion: SFM is superior to MEM in promoting HCEC survival.

INTRODUCTION

Corneal grafts can easily be preserved in organ culture, but corneal endothelial cell count declines notably about 20 – 30% during cultivation.[1] This decline continues post-surgically depending upon pre- and post-surgical risk factors.[2-5] Consequently, maintenance of graft function depends on a relatively low number of viable endothelial cells compared to healthy corneas of the same age. Hence reducing endothelial cell loss during in vitro cultivation, e.g. by ameliorating cultivation conditions, seems crucial. Most European eye banks use MEM (Minimal Essential Medium) supplemented with 2-8% fetal calf serum (FCS) as culture medium. Optimising corneal organ cultivation conditions does also include elimination of serum supplementation, because serum is a complex and varying mixture of different substances, some of them not yet determined, and underlies batch-dependent quality differences.[6,7]

Physical contact between the corneal graft and undefined, possibly pathogenic serum constituents is posing a risk to the graft recipient. Cultivation of corneal grafts in a defined serum-free medium can help avoid this risk and meets European laws and standards for handling tissue transplants.[8,9]

In previous studies we investigated the effects of nine different culture media on human corneal endothelial cell (HCEC) morphology and metabolism in HCEC cultures and whole corneas. We observed that the low nutritive content of MEM is inadequate to sustain sufficient corneal endothelial metabolic activity. We could further demonstrate that corneal grafts cultured in Human Endothelial-SFM (serum-free medium, hereafter abbreviated SFM), a humanised and fully defined culture medium, showed lowest endothelial cell loss, highest RNA synthesis rate, lowest keratan sulphate leakage, highest utilisation of glucose and lactate production.[10-13] Additional experiments demonstrated that even after prolonged organ cultivation of up to 9 weeks serum-free SFM markedly supported endothelial cell

survival and morphology, as well as keratocyte viability.[12,13] Therefore we considered Human Endothelial-SFM superior to other conventional culture media, which needed serum supplementation. Here, we compared the effects of organ culture medium on HCEC survival and protection of apoptosis and necrosis at a subcellular level.

MATERIALS AND METHODS

Cell culture and test media

The immortalized HCEC population HCEC-12, which was established by Bednarz et al. [14] and further characterized by Valtink et al. [15], was routinely grown in F99_{HCEC} (Ham's F12/Medium 199 [Biochrom AG, Berlin, Germany], 5% FCS [Invitrogen, Karlsruhe, Germany], 20µg/ml ascorbic acid [Sigma-Aldrich, Munich, Germany], 10 ng/ml human recombinant FGF-2 [Invitrogen], 20µg/ml human recombinant insulin [Sigma-Aldrich], and antibiotics [2.5µg/ml amphotericin B and 50µg/ml gentamycin, Biochrom]). For subcultivation, cells were detached with trypsin/EDTA (0.05%/0.02%, Biochrom), collected in growth medium, centrifuged at 100xg for 5min and plated at a density of 2000 cells/cm². Cultures were grown in T75 tissue culture flasks coated with 10µg/ml laminin (Invitrogen) and 10mg/ml chondroitin sulphate (Sigma) solubilised in phosphate-buffered saline (PBS) for 1h at RT. Cells were maintained at 37°C in a humidified atmosphere containing 5% CO₂. Growth medium was changed 3x per week. For experiments, medium was changed to test media 24h after plating and cells were cultured for 5days (flow cytometry) or until confluence (immunocytochemistry). Test media were MEM (Biochrom) supplemented with 2mM L-glutamine (Biochrom), antibiotics and either 2% FCS or 5% FCS, and serum-free Human Endothelial-SFM (Invitrogen) supplemented with 10ng/ml FGF-2 and either with or without

antibiotics. Medium SFM was used with and without antibiotics, because we observed a higher sensitivity of HCEC towards antibiotics in serum-free culture in preliminary experiments. HCEC cultured in growth medium F99_{HCEC} served as controls.

Induction of apoptosis and ROS production

Apoptosis via the mitochondrial pathway (intrinsic) was induced by incubating the cells with 0.5µmol/l staurosporine for 4h at 37°C. It was shown that staurosporine induces caspase-dependent apoptosis in HCEC and that markers of apoptosis such as cleaved caspase-3 and cleaved PARP are observable within less than 4h after induction.[16,17] A concentration of 0.5µmol/l sufficiently induces apoptosis in the majority of susceptible cells. Generation of ROS was induced by incubating the cells with 200µmol/l H₂O₂ for 2.5h at 37°C. Cells were photodocumented using differential interference contrast (DIC) under an IX81 fluorescence microscope (Olympus Biosystems, Hamburg, Germany) equipped with an OBS CCD FV2T camera (Olympus) driven by cell^R software (Olympus).

Flow cytometry

Flow cytometric analyses were performed using a FACS-Calibur (Becton Dickinson GmbH, Heidelberg, Germany). 10,000 cells were recorded for each analysis and calculations were performed with BD CellQuest™ software. For detection of apoptotic cells (subG1-DNA content), cells were trypsinised, pelleted at 195xg for 6min, and fixed in cold 70% ethanol for 5min on ice. 50µl of PBS containing 1µl propidium iodide solution (1mg/ml PI in H₂O; Sigma-Aldrich) and 1µl RNase (>7 U/µl, Carl Roth GmbH, Karlsruhe, Germany) were added, followed by incubation for 30min at RT in the dark. Apoptotic cells were identified by a subdiploid DNA peak (fragmented chromatin) using a 585±21nm filter (red fluorescence). Apoptotic cells were further identified by labelling chromatin after nuclear membrane destabilisation with YO-PRO-1. Cells were collected as above, resuspended in 50µl PBS with

1% horse serum (Sigma-Aldrich) containing 1µl YO-PRO-1 (100µmol/l in DMSO, Invitrogen) and 1µl PI solution, and incubated for 25min at RT in the dark. Cells were analysed using a 530±15nm filter for YO-PRO-1 (green fluorescence) and a 585±21nm filter for PI. The percentages of necrotic (PI high positive) and apoptotic (PI low positive, YO-PRO-1 positive) cells were calculated. Formation of intracellular reactive oxygen species (ROS) was measured using CM-H₂DCFDA (Invitrogen), a cell-permeant indicator which becomes fluorescent in the presence of ROS. Cells were collected as above, resuspended in 50µl CM-H₂DCFDA (10µmol/l in PBS) and incubated at 37°C for 30min in the dark. Fluorescence was recorded using a 530±15nm filter. All assays were performed at least six times, values are given as mean±SD. Statistical analysis was performed by non-parametric Mann-Whitney-U-test using SPSS (Chicago, USA) between different media (untreated cells) and between treated and untreated pairs for each medium. Alpha error adjustment by Mann-Whitney-U-test was performed with respect to the necessity of alpha error adjustment in multiple testing and with a total alpha error of 5% (0.05) for a total of 15 p-values (5 comparisons of pairs treated/untreated in the same medium and 10 comparisons of untreated cells in all five media among each other, resulting in 15 p-values). An adjusted p-value <0.05/15 (0.0033) was accepted as significant.

Intravital microscopy and immunocytochemistry

Cells were seeded into 8-chamber slides (Labtek coverglass system, Nalge Nunc International, New York, USA) and grown to confluence. Intravital staining was performed with JC-1 (20µg/ml medium; Invitrogen) at 37°C for 20-30min. JC-1 is a cationic dye that selectively accumulates within the mitochondrial matrix. JC-1 forms red fluorescent J-aggregates (emission 590 nm) in the presence of a highly negative transmembrane potential, but exists as green monomers (emission 530 nm) under depolarised conditions. The red phase of JC-1 (J-aggregate) was recorded using an excitation wavelength of 535nm and an

emission filter of 610/75nm. The green phase (monomer) was recorded using an excitation wavelength of 485nm and an emission filter of 540/50nm.

Immunocytochemical staining was performed using rabbit anti-human cleaved caspase-3 (Asp175, 1:50, Cell Signaling, Danvers, USA), rabbit anti-human Bax (1:20, clone P-19, Santa Cruz Biotechnology, Santa Cruz, USA), mouse anti-human Bcl2 (1:20, clone 124, Dako Denmark A/S; Glostrup, Denmark), and rat anti-human caspase-8 (55kD full length and cleaved, active 18kD forms of caspase-8; 1:10, clone 1H10E4H10, Zymed Laboratories, San Francisco, USA) antibodies. Secondary antibodies were FITC-conjugated goat anti-rabbit or goat anti-mouse IgG (both 1:80, Jackson ImmunoResearch Europe Ltd., Suffolk, UK), or Alexa Fluor® 488-conjugated goat anti-rat IgG (1:200, Invitrogen). Cells were washed with PBS for 5min at RT, fixed in cold acetone/methanol at 4°C for 8min, and washed again in PBS. Cells were incubated with primary antibodies at 4°C over night, washed 2x with PBS for 5min and incubated with respective secondary antibodies at 37°C for 30min (all antibodies diluted in PBS). Cells were washed 2x with PBS for 10min and nuclei were counterstained with DAPI (0.1µg/ml, Sigma-Aldrich) at RT for 5min. Samples were covered with glycerol and immediately photodocumented using an IX81 fluorescence microscope equipped with an OBS CCD FV2T camera driven by cell^R. Primary antibodies were omitted in controls. All experiments were performed at least six times.

Western blot

Cells were collected as above and lysed in 100µl ice-cold lysis buffer (60mM TRIS-HCl pH6.8, 1% SDS, 1mM NaVO₄ [Merck KG, Darmstadt, Germany]). Samples were incubated at 99°C for 5min, homogenised by sonication for 30s, and centrifuged at 14,000rpm for 3min. Protein concentration was determined using the BCA protein assay (Perbio Science, Bonn, Germany). 5x loading buffer (10% SDS, 15% glycerol, 0.01% bromophenol blue, 20% 2-mercaptoethanol, 0.3125M TRIS-HCl pH6.8 [Merck]) was added and equal amounts of protein (40µg/lane) were loaded on 10% PAA gels and electrophoresed (running buffer

0.025mol/l Tris, 0.2mol/l glycine, 0.1% SDS pH8.8 [Merck]). Protein bands were blotted onto PVDF membranes (Roth) at 4°C over night by wet western blotting (transfer buffer 0.025mol/l Tris, 0.2mol/l glycine, 20% methanol). Blots were blocked in 5% non-fat dry milk in PBS/0.1% tween-20 at RT for 1h. Membranes were then incubated with rabbit anti-human cleaved caspase-3 (1:200) or rabbit anti-human PARP p85 (caspase-cleaved 85kD fragment; 1:200, Promega Co., Madison, USA) at 4°C over night, or were incubated with mouse anti-human gamma-tubulin (1:7500, clone GTU-88, Sigma-Aldrich) at RT for 1h (all antibodies diluted in blocking solution). Detection was performed using HRP-conjugated goat anti-rabbit IgG (1:1000 for cleaved caspase-3, 1:5000 for cleaved PARP, Cell Signaling) or HRP-conjugated horse anti-mouse IgG (1:75000, Cell Signaling) diluted in blocking solution at RT for 1h and subsequent chemiluminescence using the Amersham ECL western blotting detection system (GE Healthcare, Munich, Germany) on Hyperfilm ECL (GE Healthcare).

RESULTS

Morphology and mitochondrial membrane potential

Morphology of untreated and staurosporine-treated cells was analysed by DIC microscopy after 5days cultivation in the test media (fig 1A). Untreated cells showed polygonal morphology with few vacuoles and formed regular monolayers in SFM and F99_{HCEC}, while cells in MEM were enlarged and had increased numbers and sizes of vacuoles. Furthermore, a markedly lesser cell density was observed in MEM cultures. Proliferation seemed also slightly reduced in SFM with antibiotics and cells contained more vacuoles than in SFM without antibiotics. Staurosporine induced cell shrinkage and loss of intercellular contacts in all cultures, and in MEM also caused fragmentation of the cells. Intravital staining (fig 1B)

revealed numerous mitochondria with depolarised membranes (green JC-1 fluorescence) in MEM-cultured cells irrespective of staurosporine treatment. In the other media, mitochondria of untreated cells had predominantly polarised membranes (red JC-1 fluorescence), and became depolarised only after staurosporine treatment.

Flow cytometric analysis of apoptosis, necrosis and oxidative stress

FACS analysis revealed that HCEC could be divided into four subpopulations after staurosporine treatment, namely healthy cells (PI and YO-PRO-1 negative), YO-PRO-1 positive (apoptotic) cells, highly PI-positive (necrotic) and PI low-positive (apoptotic, see Zamai et al. 1996 and 2001) cells.[18,19] The number of healthy cells in untreated cultures was significantly higher in both SFM media ($95.6\pm1.6\%$, $95.6\pm1.9\%$) than in F99_{HCEC} ($87.5\pm2.0\%$) and MEM media ($79.1\pm2.8\%$ and $81.9\pm4.4\%$), and was also significantly higher in F99_{HCEC} than in MEM+2% FCS. Adding staurosporine significantly reduced the number of healthy cells in F99_{HCEC} ($66.2\pm3.5\%$) and MEM media ($28.6\pm2.4\%$ and $22.4\pm0.8\%$), but not in SFM media ($93.0\pm3.3\%$ and $94.4\pm2.3\%$) (fig 2A). The number of necrotic cells in untreated controls was significantly lower in both SFM media ($2.1\pm1.3\%$ and $1.7\pm1.1\%$) than in the other media (F99_{HCEC} $7.2\pm1.2\%$; MEM $13.0\pm2.5\%$ and $10.8\pm3.6\%$). After staurosporine treatment, the number of highly PI-positive cells increased significantly in F99_{HCEC} ($14.3\pm2.3\%$) and in MEM ($33.2\pm3.6\%$ and $33.9\pm3.1\%$) compared to their untreated counterparts (SFM $2.7\pm2.5\%$ and $1.8\pm1.0\%$, not significant), (fig 2B). The number of YO-PRO-1 positive cells increased significantly after staurosporine treatment in F99_{HCEC} from $1.8\pm0.5\%$ to $11.7\pm3.2\%$ and in both MEM media from $1.0\pm0.2\%$ to $21.1\pm6.4\%$ and from $1.3\pm0.3\%$ to $31.8\pm4.3\%$, but not in SFM media (from $0.8\pm0.2\%$ to $2.1\pm1.1\%$ and from $0.9\pm0.5\%$ to $1.9\pm1.0\%$) compared to untreated controls (fig 2C). The number of PI low positive cells in untreated controls was significantly fewer in SFM media ($1.5\pm0.4\%$ and $1.8\pm0.9\%$) than in the other media (F99_{HCEC} $3.5\pm0.8\%$; MEM $6.8\pm0.3\%$ and $6.0\pm0.7\%$) and was also significantly lower in F99_{HCEC} compared to MEM+2% FCS. Staurosporine-induced

cell stress was significantly higher in both MEM media ($17.1\pm 4.1\%$ and $11.9\pm 0.8\%$) and in F99_{HCEC} ($7.8\pm 1.8\%$), but not in SFM media ($2.2\pm 0.6\%$ and $1.9\pm 0.5\%$) (fig 2D). The number of cells with fragmented chromatin (subG1-DNA-content) was significantly lower in untreated SFM cultures ($4.2\pm 1.0\%$ and $4.4\pm 1.8\%$) than in untreated MEM media cultures ($14.2\pm 3.7\%$ and $8.6\pm 1.8\%$). Staurosporine-induced DNA fragmentation was significantly higher in MEM media ($41.9\pm 2.2\%$ and $48.0\pm 2.9\%$) and in F99_{HCEC} ($18.2\pm 4.5\%$) compared to their untreated counterparts, but not in SFM media ($8.7\pm 3.5\%$ and $7.7\pm 3.1\%$) (fig 2E). ROS production in untreated control cultures was significantly lower in MEM+5% FCS (32.1 ± 2.7) than in the other media (F99_{HCEC} 98.2 ± 45.7 ; MEM+2% FCS 44.0 ± 5.4 ; SFM 86.2 ± 19.7 and 55.9 ± 20.2) and was also significantly lower in MEM+2% FCS than in SFM. Cells cultured in MEM or F99_{HCEC} showed a significantly higher ROS production after treatment with H₂O₂ (MEM 192.1 ± 51.3 and 97.4 ± 7.3 , F99_{HCEC} 238.2 ± 50.6) compared to their untreated counterparts, but not in SFM media (116.7 ± 14.8 and 96.9 ± 9.7) (fig 2F).

Immunocytochemical analysis of apoptotic parameters

Upon staurosporine treatment, cells in MEM and F99_{HCEC} cultures showed stronger staining for cleaved caspase-3 (fig 3A), cleaved caspase-8 (fig 3B), Bax (fig 4A), and Bcl-2 (fig 4B), and to some extent stronger staining for cleaved caspase-8 in cells cultured in SFM with antibiotics. The staining pattern for caspase-8 and Bax changed from a single perinuclear, punctate localisation in untreated controls to a diffuse cytoplasmic appearance. Especially cells cultured in MEM media were highly susceptible to apoptosis, indicated by pronounced Bcl-2 and Bax positivity already in untreated cultures. Interestingly, cells cultured in SFM seemed less affected by staurosporine, since staurosporine failed to induce up-regulation of Bcl-2 and the other tested apoptosis markers. Omission of the primary antibodies in negative control stainings confirmed specificity of the staining (fig 5). Western blotting (fig 3C) revealed more cleaved PARP and cleaved caspase-3 after staurosporine treatment in all cultures, with cells cultured in MEM media and F99_{HCEC} producing markedly higher amounts

of cleaved PARP and cleaved caspase-3 compared to cells cultured in SFM media. Besides, cleaved caspase-3 could also be detected in untreated cells cultured in medium F99_{HCEC}, and cleaved PARP could also be detected in untreated cells cultured in MEM+5% FCS.

DISCUSSION

Endothelial cell density and morphology are the two main criteria for evaluating the quality of corneal grafts before keratoplasty, both being influenced by storage conditions. European eye banks favour long-term storage at 30-37°C, and most commonly a MEM-based medium is used. MEM has a reduced nutrient content and needs to be supplemented with fetal calf serum to maintain cell survival. Improvements in cell and organ culture techniques and updated legal guidelines for handling tissues for transplantation require serum replacement by defined, ideally humanised, factors. Previous investigations showed that metabolic requirements of corneal endothelial cells are quite particular and are only inadequately met by MEM in corneal endothelial cell and corneal organ culture.[10-13,20]

The results obtained here support these former reports and clearly show that nutrient deficiency in MEM does not only lead to a reduced metabolic and proliferative activity, but results in an elevated susceptibility to apoptotic cell death in HCEC. Dramatic morphological changes of HCEC cultured in MEM, like pronounced vacuolisation, are key features of a reduced viability and are already visible in untreated control cultures. Signs of cell death, such as chromatin fragmentation, mitochondrial membrane depolarisation, and labelling with PI and YO-PRO-1, can be detected in MEM-cultured HCEC even without exogenously inducing apoptosis. Adding the apoptosis-inducer staurosporine to MEM-cultured cells

resulted in a marked increase of these indicators and also in an up-regulation of the apoptotic markers caspase-3 and PARP and hence also their cleaved forms, as detected by western blotting. Furthermore, a higher staining intensity for Bax and cleaved caspase-8 was noted irrespective of the amount of serum added. This increase in staining intensity was accompanied by cytoplasmic spreading of the formerly single punctate pattern in untreated and healthy cells, which strongly resembled the staining pattern of p53, a transcription factor that regulates transcription of molecules associated to the apoptotic pathways and localises in close proximity with caspase-8 and Bax, amongst others, in healthy cells. Upon induction of apoptosis caspase-8 is up-regulated, cleaved and distributed into the cytoplasm. Likewise, Bax is up-regulated, oligomerised and also distributed to the cytoplasm.[21-23] Up-regulation of Bcl-2, an anti-apoptotic protein which is expressed in organ-cultured human cornea [24,25], may be regarded as a protective mechanism sustained by the cells. For example, the higher amount of Bcl-2 in untreated cultures kept in MEM+2% FCS compared to the other untreated cultures may indicate that nutrient supply in this medium is insufficient and the cells up-regulate Bcl-2 to counteract death by starvation. Contrasting, HCEC in SFM appeared to be protected from cell death despite the lack of serum, as the examined apoptotic factors were significantly lower under control conditions and did not increase significantly after staurosporine administration. It also appeared that addition of antibiotics, which can be noxious under serum-free conditions, was well tolerated in SFM.

ROS are formed as a physiological product of respiration, hence higher ROS production in cells in SFM and F99_{HCEC} under control conditions reflects a higher metabolic activity. ROS production does not increase significantly under administration of H₂O₂ in SFM, indicating that HCEC in SFM are protected against oxidative stress by utilising anti-oxidative or compensatory mechanisms. Contrasting, HCEC in MEM form only little ROS under control conditions, but increase ROS production more than double under H₂O₂, pointing to a reduced respiratory and metabolic activity and insufficient anti-oxidative capacity of the cells. This is

further substantiated by the observed depolarised mitochondrial membranes in MEM-fed control cultures.

In summary, the results show that cells cultured in MEM media seem susceptible to cell death already in the absence of exogenous noxious stimuli, while HCEC cultured in SFM seem to be protected from cell death even when the apoptosis-inducer staurosporine is added. The nutritive conditions in MEM therefore seem to be insufficient to sustain adequate compensatory mechanisms against oxidative cell stress. Thus Human Endothelial-SFM is superior to conventionally used MEM in promoting HCEC survival in vitro cell cultures. It is known that cultured cells react more sensitive to stimuli than cells in situ, and it can be assumed that the microenvironment of corneal endothelial cells in situ may contain protective factors that prevent endothelial cell death in MEM-based organ cultured donor corneas in such an extensive way as was observed in our experiments. However, based on our results and with respect to european laws and guidelines on tissue handling we suggest that the methods of organ cultivation of donor corneas should be improved. A multi-centric clinical trial to transplant SFM-stored corneas vs. MEM-stored corneas was initiated in Germany (registered at clinicaltrials.gov, identifier NCT00623584).

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Acknowledgements

We wish to thank Ms B. Rost, Ms S. Bramke and Ms A. Neisser for technical assistance.

LEGENDS

Figure 1 HCEC morphology and polarization state of mitochondrial membranes. Cells were cultured in the respective test media for 5d, and then part of each culture was treated with 0.5 μ mol/l staurosporine for 4h at 37°C to induce apoptosis. (A) Morphology of the cells viewed by DIC microscopy. (B) Intravital staining with JC-1 indicates mitochondria with predominantly polarised (red) membranes in untreated controls in SFM and F99_{HCEC}, and depolarised (green) mitochondrial membranes in untreated MEM controls and in all media after addition of staurosporine. Scale bar 100 μ m.

Figure 2 Flow cytometric analysis of HCEC. Cells were cultured in the respective test media for 5d, then part of each culture was treated with 0.5 μ mol/l staurosporine for 4h at 37°C to induce apoptosis (A-E) or was treated with 200 μ mol/l H₂O₂ for 2.5h at 37°C to induce oxidative stress (F). (A) Number of healthy (PI-/YO-PRO-1 negative) cells, (B) number of necrotic (PI-positive) cells, (C) number of apoptotic (YO-PRO-1 positive) cells, (D) number of apoptotic (low PI-positive) cells, (E) number of cells with fragmented nuclei (sub G1 DNA-content), (F) ROS production was visualised by means of CM-H₂DCFDA staining, fluorescence intensity is given in arbitrary units. Data are given as mean \pm SD (* p<0.05). White bars: untreated control cultures, grey bars: treated with staurosporine, black bars: treated with H₂O₂.

Figure 3 Determination of cleaved caspase-3, full length and cleaved caspase-8 and cleaved PARP. Cells were cultured in 5 test media for 5d and part of each culture was treated with 0.5 μ mol/l staurosporine for 4h at 37°C to induce apoptosis. Cells were then fixed and stained with antibodies against cleaved caspase-3 (A) and full length and cleaved caspase-8 (B). Scale bar 100 μ m. (C) Western blot analysis of cleaved caspase-3 and cleaved PARP (loading control: γ -tubulin).

Figure 4 Determination of Bax and Bcl-2. Cells were cultured in 5 test media for 5d and part of each culture was treated with 0.5 μ mol/l staurosporine for 4h at 37°C to induce apoptosis. Cells were then fixed and stained with antibodies against Bax (A) and Bcl-2 (B). Scale bar 100 μ m.

Figure 5 Negative control stainings of HCEC cultured in MEM+2% FCS and treated with staurosporine for 4h at 37°C to induce apoptosis. The primary antibodies were omitted. (A) cleaved caspase-3, (B) full length and cleaved caspase-8, (C) Bax, (D) Bcl-2; scale bar 100µm.

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COMPETING INTERESTS

Competing Interest: None declared.

FUNDING

no funding

COLOUR FIGURES AND COSTS

This manuscript contains 3 colour figures. The costs for printing the 3 colour figures will be taken over by

Institute of Anatomy
Medical Faculty at the TU Dresden
Fetscherstr. 74
01307 Dresden
Germany

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