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THE m.3243A>G mtDNA MUTATION IS PATHOGENIC IN AN *IN VITRO* MODEL OF THE HUMAN BLOOD BRAIN BARRIER

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Abstract

MELAS is a common mitochondrial disease frequently associated with the m.3243A>G point mutation in the tRNA^{Leu(UUR)} of mitochondrial DNA and characterized by strokelike episodes with vasogenic edema and lactic acidosis. The pathogenic mechanism of stroke and brain edema is not known. Alterations in the blood brain barrier, (BBB) caused by respiratory chain defects in the cortical microvessels could explain the pathogenesis. To test this hypothesis we developed a tissue culture model of the human BBB. The MELAS mutation was introduced into immortalized brain capillary endothelial cells and astrocytes. Respiratory chain activity and transendothelial electrical resistance, TEER was measured. Severe defects of respiratory chain complex I and IV activities, and a moderate deficiency of complex II activity in cells harboring the MELAS mutation were associated with low TEER, indicating that the integrity of the BBB was compromised. These data support our hypothesis that respiratory chain defects in the components of the BBB cause changes in permeability.

Keywords

Cell culture model; blood brain barrier; MELAS; stroke; endothelial cells; astrocytes

1. Introduction

Mitochondrial encephalopathy, lactic acidosis and strokelike episodes (MELAS), one of the most common and widely studied maternally inherited mitochondrial diseases, is frequently associated with the m.3243A>G point mutation in the mitochondrial tRNA^{Leu(UUR)} gene. The clinical phenotype is multisystemic, but the triad of lactic acidosis, seizures, and strokelike episodes remains crucial to the diagnosis and reflects the complex and unique pathogenesis of this syndrome (Finsterer, 2007). The levels of ventricular CSF lactate correlate with the severity of neurological impairment (Kaufmann et al., 2004). Morphologically, muscle biopsies show ragged red fibers (RRF) due to mitochondrial proliferation, with partial cytochrome *c* oxidase (COX) deficiency. Marked proliferation of mitochondria is also present in blood vessels (strongly SDH staining blood vessels, SSVs) (Hasegawa et al., 1991, Bonilla et al., 1992). Our studies of cybrids showed that severe defects in protein synthesis and respiratory chain function

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segregate with the mutation, although the pathogenic threshold is high, more than 90% mutant mtDNAs are required to cause dysfunction (King et al., 1992). However, most MELAS patients have well below 95% mutant mtDNA, suggesting that the data from cybrid studies may not be directly extrapolated to the clinical status. Moreover, the pathogenic mechanism of strokes and vasogenic edema cannot be explained by the available data. Without a better understanding of pathogenesis, rational therapeutic intervention has not been possible.

The strokes, non-ischemic in origin and therefore called ‘stroke-like episodes’, are at least partially reversible, and do not conform to distribution of large cerebral arteries, but rather affect small arterioles and capillaries of the cortex, while sparing the adjacent white matter (Sproule and Kaufmann, 2008). The recurrent strokes are associated with vasogenic edema, as demonstrated by MR diffusion weighted imaging (DWI) studies, suggesting that they may be due to increased permeability in the BBB, perhaps caused by mitochondrial dysfunction in the endothelium of cerebral small vessels (Yoneda et al., 1999, Ohshita et al., 2000). Furthermore, the accumulation of ventricular lactate indicates severe energy failure in the brain due to mitochondrial dysfunction and acute hypoxia during stroke-like episodes. Translational defects of the mitochondrial respiratory chain (RC) subunits and pathological alterations in the microvasculature and in BBB components have been documented in patients with MELAS, thus supporting the notion that BBB permeability may be increased due to mitochondrial respiratory failure in the cortical microvasculature (Tanji et al., 2001). To analyze the functional status of the BBB in MELAS, we introduced mitochondria with 97% of the m.3243A>G mtDNA mutation into normal endothelial cells (EC), (hCMEC/D3), and into normal astrocytes (IHFA), and compared the mitochondrial function in the component cells of the normal and MELAS BBB (Weksler et al., 2005, Su et al., 2003). Our data reveal severe defects of RC complexes in immortalized endothelial cells and astrocytes as well as in primary astrocytes harboring the m.3243A>G mutation. Furthermore, the defects in EC cells with the MELAS mutation correlate with lower transendothelial electrical resistance (TEER) indicating increased permeability of the EC. Co-cultures of EC and astrocytes with and without the MELAS mutation will be constructed to evaluate paracellular permeability and transport of lactate and water.

2. Materials and methods

2.1. Cell culture

2.1.1. Normal fetal astrocytes—Immortalized human fetal astrocytes (IHFA) expressing the hTERT gene were obtained from Dr. Paul Fisher, Virginia Commonwealth University School of Medicine, VA. This cell line expresses the astrocyte marker, GFAP (Su et al., 2003). Cell lines were grown in DMEM supplemented with 10% fetal bovine serum (FBS) at 37°C in a 95% air, 5% CO₂ humidified incubator.

2.1.2. Normal brain capillary endothelial cells—hCMEC/D3 cells immortalized by lentiviral transduction of SV-40 large T-antigen and hTERT were obtained from Dr. Babette Weksler, Weill Medical College of Cornell University, New York, NY. This cell line expresses markers characteristic of EC, such as tight junction proteins, adhesion proteins, chemokine receptors and exhibits drug exclusion properties. The cells were grown in endothelial growth medium-2 (EGM-2MV; Clonetics; Cambrex Biosciences) supplemented with vascular endothelial growth factor, insulin-like growth factor-1, epidermal growth factor, basic FGF (bFGF), hydrocortisone, ascorbate, gentamycin and 2.5% FCS, as described (Weksler et al., 2005).

2.1.3. MELAS Astrocytes—Normal fetal astrocytes immortalized with hTERT were repopulated with mitochondria harboring 97% levels of the m.3243A>G mtDNA mutation by

our published method (Sobreira et al., 1999). Rhodamine 6G (R6G) toxicity varies with different cell types. Therefore, we performed a preliminary titration of R6G concentration and time of treatment to determine optimum conditions. IHFA fetal astrocytes were treated with 3 µg/ml R6G for 5 days to render the mitochondria non-viable, and subsequently fused with enucleated cytoplasts from cybrids harboring 98% levels of the m.3243A>G MELAS mutation (RN164) (King et al., 1992), using polyethylene glycol (PEG 50%). The cells were propagated in DMEM containing 10% FBS. In the absence of a selectable marker, we picked ~75 colonies and screened them for, (1) the m.3243A>G mutation by RFLP analysis; and (2) the expression of GFAP, an astrocyte marker, by immunocytochemistry. Two clones, 16 and 21, which expressed both GFAP and the m.3243A>G mutation at 97%, were selected for further studies (Fig. 1. scheme).

2.1.4. MELAS Endothelial cells—Normal brain capillary EC (hCMEC/D3) were treated with 0.75 µg/ml R6G for 3 days and fused with enucleated RN164 cytoplasts as described above to transfer mitochondria with the MELAS mutation. The fused cells were grown in EC culture medium and subcloned. Fifty colonies were picked and screened for the m.3243A>G mutation by RFLP analysis and for the Von Willebrand factor and Claudin-1 by immunocytochemistry. Clones 2 and 10 expressed both the mutation and the EC markers and were selected for further studies. (Fig. 1).

2.1.5. MELAS cybrid, RN164—RN164, a cybrid cell line with the osteosarcoma (143B) nuclear background and containing 98% levels of the m.3243A>G mtDNA mutation, was generated and characterized as described previously (King et al., 1992).

2.1.6. Primary astrocyte cultures—Normal primary human astrocytes were obtained from Cambrex Corporation (East Rutherford, NJ, USA) and cultured according to the manufacturer's instructions.

Brain tissue was obtained 3 hours postmortem from a patient with MELAS and primary astrocytes were isolated and cultured by the method described by Murakami et al., (Murakami et al., 1999). Briefly, cortical tissue was dissected in DMEM (Invitrogen Life Technologies, Carlsbad, CA) containing 10% calf serum (CS), 4 g/L glucose, 100 units/ml penicillin, and 100 mg/ml streptomycin, rinsed in medium without serum, and treated with 0.25% trypsin for 10 minutes at 37°C. The cells were dissociated by serial extrusion through stainless steel mesh, 230 µm pore size, 60-mesh screen, followed by 140 µm pore size, 100-mesh screen into a 100 mm² dish containing DMEM. The cell suspension was centrifuged, the pellet washed three times and finally resuspended in DMEM containing 5% CS, 4 g/L glucose, 0.05% NaHCO₃, 292 µg/ml L-glutamine, 100 units/ml penicillin, 100 mg/ml streptomycin, and plated in 100 mm² tissue culture dishes. The cultures were subcloned and single colonies were picked and grown. Clone MAG1 was selected for further studies.

Normal and MELAS primary astrocyte cultures were plated on glass coverslips for immunocytochemistry with antibodies to the astrocyte marker GFAP, and in 100mm² dishes for quantitation of the m3243A>G mutation by RFLP analysis.

2.2. RFLP analysis

Quantitation of the m.3243A>G mtDNA mutation was performed by RFLP analysis on the RN164 cybrids; normal astrocytes (IHFA) and MELAS astrocyte clones 16 and 21; normal primary astrocytes and MELAS primary astrocyte clone, MAG1; normal endothelial cells hCMEC/D3 and MELAS EC clones 2 and 10 as described previously (King et al., 1992). Briefly, PCR amplification was performed with the last hot cycle using ³²P nucleotide,

followed by digestion with *Hae III*. The bands were separated on a 2% acrylamide gel and quantitated on Phosphorimager.

To detect changes in the mutant load during analyses, PCR amplification was performed with cold nucleotides, digested and separated on an agarose gel and visualized by staining with ethidium bromide.

2.3. mtDNA quantitation

The abundance of mtDNA copy number was determined by real-time PCR using SYBR Green detection with an Applied Biosystems 7300 Real-time PCR System (Invitrogen Life Technologies, Carlsbad, CA) as described (Partridge et al., 2007). The amplified products were a 207-bp fragment of the nuclear encoded 18S rRNA gene and a 199-bp fragment of the mtDNA-encoded 12S rRNA gene. The primers were as follows, 18S sense- AAGCTTGCGTTGATTAAGTCC; 18S antisense- TAATGATCCTTCCGCAGGTTC; 12S sense- GGGTTGGTAAATTTTCGTGCCAGC; and 12S antisense- CCCAGTTTGGATCTTAGCTATC. All reactions were done in triplicate. PCR conditions were as follows, 95°C for 15 min followed by 40 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 30 s. Relative quantification of mtDNA/nDNA ratio was determined by the comparative threshold cycle (CT) method, as described (Shao et al., 2006).

2.4. Immunocytochemistry

Immunocytochemical analysis of primary normal and MELAS astrocytes was performed. Cells grown on glass coverslips, fixed and permeabilized with 4% (w/v) paraformaldehyde were incubated with monoclonal antibodies to COX subunit II (Molecular probes, OR) or to the E2 subunit of pyruvate dehydrogenase (PDH) (The Binding site, UK), followed by FITC labeled secondary antibody (GE healthcare Biosciences Corp., Piscataway, NJ, USA) and examined with a Zeiss Axiovert 200M, inverted fluorescent microscope (Carl Zeiss, Göttingen, Germany), using epi-illumination, as described (Davidson et al., 2005).

2.4. Histochemistry

To evaluate mitochondrial function, COX and SDH histochemistry were performed as described (Salviati et al., 2002). After incubation with the substrates, the coverslips were rinsed in PBS, mounted in glycerin-gelatin, and examined with a Zeiss microscope (Carl Zeiss, Göttingen, Germany) with brightfield optics.

2.5. Biochemistry

We performed biochemical analyses of the RC complexes: rotenone sensitive NADH-ubiquinone oxidoreductase (Complex I), SDH (Complex II), COX (Complex IV), and of the mitochondrial matrix enzyme citrate synthase (CS) on freshly harvested and homogenized cell pellets (Salviati et al., 2002, Pallotti et al., 2004). All assays were done in duplicate on three independently harvested samples.

2.6. Transendothelial electrical resistance (TEER)¹⁰, section 2.7

For TEER measurements, normal EC cells were seeded at a density of 5×10^5 and MELAS EC at a density of 7×10^5 on Transwell inserts, 12 mm diameter, growth area 1 cm² (Corning Costar Corporation, Cambridge, MA) with 4 µm size pores, coated with collagen Type IV, and grown to confluence. The difference in plating density was to ensure that homogeneously confluent monolayers were similar in the normal and MELAS cultures. TEER was measured at various time points using an EndOhm-12 chamber and an EVOM voltohmmeter (World Precision Instruments, Sarasota, FL, USA) as described (McAllister et al., 2001). The apparatus consists of a chamber and a fitted cap with electrodes. The inserts with the EC are placed in

the chamber and voltage is applied across the cell monolayer resulting in resistance to current flow, which is read off the voltohmmeter. The resistance of a blank filter is also measured. TEER ($\Omega \cdot \text{cm}^2$) is calculated from the displayed electrical resistance on the readout screen of the voltohmmeter, multiplied by the filter surface area, and corrected for blank filter resistance. Readings are taken in triplicate from replicate cultures. Increasing TEER values are an indication of tight junction formation.

2.7. Statistical analyses

Standard deviation (SD) was analyzed using the statistical analysis software, SAS. Differences in the distributions between wild type and mutant cells were assessed using (2-sided) Wilcoxon Rank Sum tests. Exact P-values of ≤ 0.05 was considered statistically significant.

3. Results

3.1. RFLP analysis

Wild-type (WT) astrocytes (IHFA) and MELAS astrocytes (Clone 16 and 21) as well as WT EC (hCMEC/D3) and MELAS EC (Clones 2 and 10) were checked for repopulation with the MELAS mutation by RFLP analysis. Quantitation of the mutation by phosphoimager revealed 97% mutant mtDNA in all the mutant clones at the time of repopulation (gel not shown). These data confirm that the R6G treatment and subsequent fusion with “MELAS cytoplasts” were successful.

RFLP analysis done during the time of the histochemical, biochemical and TEER analyses confirmed that there were no changes in the mutation load between the time of repopulation and the time of analyses. Ethidium bromide stain of the *Hae III* digested PCR products (Fig. 2A, B) shows that the wild type EC and astrocytes (lanes 6, 7 and 11) have the WT band of 192 kb; the MELAS EC clones 2 and 10 (lanes 4 and 5), MELAS astrocyte clones 16 and 21 (lanes 8 and 9) and the MELAS cybrids, RN 164, have the “diagnostic” MELAS bands of 97 and 72 kb.

Normal primary astrocytes and MELAS primary astrocytes cultured from autopsy brain were also analyzed. As shown in Fig 2B, the 97 and 72 kb bands diagnostic of the m.3243A>G mutation are present in MELAS primary astrocytes, (MAG1), in addition to low levels of the WT band corresponding to 192 kb (lane 1); the WT band of 192 kb is present in normal primary astrocytes (lane 2). Quantitation by phosphoimager (Biorad, USA) revealed a mutation load of 89% in MAG1 cells.

3.2. mtDNA copy number

We assessed mtDNA copy number by comparing the ratios of mtDNA to nDNA amplified by quantitative RT PCR to verify that mtDNA levels were normal in cell lines repopulated with exogenous mitochondria harboring the m.3243A>G mutation. As shown in Fig. 3, the mtDNA/nDNA ratios of the clones harboring the mutation were normalized to their corresponding WT cell lines. The ratios in hCMEC/D3 clones 2 and 10 were similar to those in the hCMEC/D3 parental cell line and the ratios in IHFA clones 16 and 21 were slightly lower than those of the IHFA WT cell line. Statistical analysis indicates that there were no significant changes in the mtDNA copy number during repopulation.

3.3 COX histochemistry of normal and MELAS EC and astrocytes

We also compared the RC status in WT EC and astrocytes and in the clones harboring 97% of the MELAS mutation using histochemical reactions for COX and SDH. Staining for COX was decreased in both MELAS EC clones (2 and 10) compared to WT cells. Interestingly, SDH staining was also low, as shown in Fig. 4A. Similarly, COX histochemistry of astrocytes was

reduced in MELAS astrocyte clones 16 and 21 as compared to WT. SDH histochemistry was reduced in one of the MELAS clones (Fig.4B).

3.4. COX biochemistry of normal and MELAS astrocytes and EC

To confirm the histochemical data, we performed biochemical assays of RC enzymes, complex I, complex II (SDH), complex IV (COX) and of the matrix marker enzyme citrate synthase (CS) in WT and MELAS EC and astrocytes. As shown in Table 1, complex I activities were significantly decreased in astrocyte clones 16 and 21 (22–60% of WT values) and even more so in EC clones 2 and 10 (5–40% of WT values). COX activities were also significantly reduced in the MELAS clones (52–67%) though not as severely as complex I. CS levels were not significantly altered in either the EC or the astrocyte clones. When normalized to CS, a mitochondrial matrix marker, the pattern of enzyme deficiencies described above was confirmed in both EC and astrocytes, indicating that the impairment of RC activity is real and not related to changes in mitochondrial mass. The defects of complex I and complex IV in astrocytes and EC harboring 97% levels of the m.3243A>G mutation are similar to those found in muscle, cybrid cell lines, and other tissues from MELAS patients (Sproule and Kaufmann, 2008). Interestingly, SDH levels in the MELAS astrocyte clone 16 and in both EC clones were also slightly lower (64–77%) than in the WT counterparts.

3.5. Studies of normal and MELAS primary astrocytes

We chose to study immortalized astrocytes and EC cell lines because of their robust growth properties and stability on prolonged passaging. To confirm that immortalized cells were similar to primary cells in their expression of RC abnormalities, we studied both RC histochemistry and translation of RC subunits by immunocytochemistry.

In primary astrocytes, MAG1, isolated from the brain of a MELAS patient, histochemical staining for COX was reduced whereas SDH staining was normal (Fig.5e, g), similar to our observations in immortalized astrocytes, compared to normal primary astrocytes IHFA, (Fig. 5f, h). Steady state levels of mtDNA-encoded COX subunit II was also reduced by immunocytochemistry (Fig. 5a), compared to normal primary astrocytes (Fig. 5b). In contrast, staining for the nuclear encoded E2 subunit of PDH used as a control was normal in both the MELAS and normal primary astrocytes (Fig. 5c, d). These results indicate that both RC function and translation of mitochondrially encoded subunits are defective in primary astrocytes with the m.3243A>G mutation and validate our data from transformed cells.

3.6. Transendothelial electrical resistance (TEER)

TEER was measured in hCMEC/D3 WT and mutant clones up to 17 days. As shown in Fig. 6, TEER values gradually increased in both normal and MELAS EC with days in culture, reaching peak values at about 9 days in culture, indicating that tight junctions were being assembled. However, the TEER was consistently lower in MELAS EC as compared to WT EC and never reached the WT values. Clone 2 readings rapidly declined, indicating increased permeability of the EC. TEER readings of both clone 2 and 10 were 70–80% of that of WT cells and were lower at every time point analyzed. The readings are consistent with TEER values reported for transformed cells (Kannan et al., 2000, Tan et al.). These results are an indication that the BBB may be perturbed in cells with the MELAS mutation and provide an important proof of principle for the use of the *in vitro* model for the analysis of the BBB.

4. Discussion

MELAS caused by the m.3243A>G mtDNA mutation is perceived as a vascular disease based on the mitochondrial proliferation in the microvasculature of the cortex (SSV), the relative sparing of the white matter, and the recurrence of strokes that do not appear to be ischemic,

but rather due to a metabolic angiopathy. The pathogenic mechanism of the stroke-like episodes and the accompanying vasogenic edema is attributed to mitochondrial dysfunction in the cell components of the BBB, which include endothelial cells, astrocytes, and the neurons in the brain microvessels. This plausible explanation has not yet been verified experimentally. The absence of animal models of MELAS, specifically, and of mtDNA-related diseases in general, makes this hypothesis difficult to be tested. Thus, we have to rely on autopsy tissue samples and on tissue culture systems to evaluate the pathogenic mechanism of strokes in MELAS. We focused on developing a cell culture model of the BBB to answer the following questions, (i) does the m3243A>G mtDNA mutation in MELAS patients segregate with RC dysfunction in the constituent cell types of the BBB? and (ii) if so, do the mitochondrial defects correlate with altered features of the vascular endothelial cells and the astrocytes?

For this purpose, we used as controls immortalized normal brain capillary endothelial cells, hCMEC/D3, and immortalized normal fetal astrocytes, IHFA. We eliminated the endogenous mitochondria from these cell types and repopulated them with mitochondria harboring 97% levels of the MELAS mutation. The MELAS genotype was quantitatively transferred to the target cells and the WT and mutant cell lines contain the expected genotypes. Furthermore, the repopulation did not result in significantly lower copy number of mtDNA in the mutant clones of either EC or of astrocytes or to contamination with WT mtDNA. Therefore, the RC dysfunction in cultured EC or astrocytes is clearly due to the m3243A>G mutation and *not* to lower mtDNA or mitochondrial copy number or to mitochondrial mass as indicated by normal citrate synthase activity.

4.1. Respiratory chain function, TEER and BBB

Histochemical analysis of WT and MELAS EC and astrocytes revealed severe deficiency of COX in both types of cells and lower than normal SDH staining in both EC clones and in one astrocyte clone, indicating deficiency of the RC in the mutant cells. These results were corroborated by biochemical assays of RC enzymes, which showed markedly decreased complex I and IV activity in the clones with the mutation. These findings are similar to those we obtained in the parental MELAS cybrid RN164, from which mutant mitochondria were transferred to EC and astrocytes (King et al., 1992). In a study of brain regions from two patients with MELAS, Betts et al (2006) found good correlation between the highest mutation load and the most severe COX deficiency in the walls of the leptomeningeal and cortical blood vessels, which led the authors to conclude that vascular mitochondrial dysfunction is directly related to the pathogenesis of stroke-like episodes in MELAS (Betts et al., 2006). Therefore, our finding of RC deficiency in the EC and astrocytes establishes a cause-effect relationship of the mutation to the pathogenesis of the strokes, due to a breakdown of the BBB.

Defects of complex I and IV are common in patients with MELAS (King et al., 1992). Unexpectedly, SDH levels (Complex II) were also low in these clones. Because all the four subunits of complex II are encoded by the nuclear DNA, SDH activity is usually normal in cybrids harboring the MELAS mutation. Low SDH levels observed in our study may result in decreased electron transfer to complex III resulting in lower ATP production. Low SDH levels may result in increased superoxide levels by depleting the electrons in the ubiquinone pool. (Rustin et al., 2002). Besides, elevated levels of superoxides are also produced by the flavin radicals of complex I in patients with Complex I deficiency and in tissues of trans-mitochondrial mice with the m.3243A>G mutation (Raha and Robinson, 2000, Li et al., 2008). Studies using the m.3243A>G cybrids have demonstrated that RC defects caused by the high levels of mutation have resulted in increased oxidative stress (Wong and Cortopassi, 1997, Pang et al., 2001). Therefore, the combined effects of defective complex I, II, and IV may (1) impair ATP synthesis in both EC and astrocytes and (2) may increase ROS production, both of which have a bearing on BBB permeability as indicated by TEER readings.

4.2. Transendothelial electrical resistance (TEER)

Our data reveal consistently lower TEER values in MELAS EC than in the WT EC clones, which reflect compromised permeability. Notably, the permeability of the BBB is tightly regulated by ATP-dependent junctional proteins and carrier-mediated transport systems (Sandoval and Witt, 2008). It is also known that the mitochondrial content in EC of brain capillaries is higher than in other tissues, indicating higher metabolic activity (Oldendorf et al., 1977). Therefore, low ATP levels in EC can affect the integrity of the BBB, and are compounded by similar effect by increased ROS. Studies have shown that disruption of the BBB is an early target for superoxides in rats subjected to cryogenic brain injury and that superoxides play an important role in cerebral edema following focal cerebral ischemia (Ikeda et al., 1994, Kondo et al., 1997). Lagrange et al using an *in vitro* model of the BBB have demonstrated that menadione induced generation of superoxides significantly increased EC permeability in a dose dependent manner, which was reversed by pre-incubation with superoxide dismutase (Lagrange et al., 1999). In the light of the above data, it is reasonable to speculate that the RC defect in mutant EC cells is likely to cause low ATP levels and increase in superoxides, which may subsequently result in the breakdown in the BBB. The TEER data though preliminary, provides clues to the cause and effect relationship of the RC defect with the BBB status.

4.3. Analysis of primary astrocytes

Primary astrocytes cultured from a MELAS brain also revealed low COX by histochemistry compared to normal primary astrocytes. These data provide evidence that the immortalized astrocytes used in this study maintain a phenotype similar to that of primary cells. The ease of passaging and robust growth properties make these immortalized cells good candidates for further analysis of BBB permeability in MELAS. Additionally, the immunocytochemical expression of the mitochondrially encoded COX II subunit was severely decreased compared to the expression of the nuclear encoded pyruvate dehydrogenase E2 subunit indicating that the mutation causes a shutdown of mitochondrial translation in the component cells of the BBB. A similar study by Tanji et al (2001) documented that the translation of COX II was impaired in the cortical microvasculature of a MELAS brain, demonstrating that there is a consistency of results extending from tissue to primary cells to immortalized cell lines, which tends to validate extrapolation (Tanji et al., 2001).

5. Conclusions

EC and astrocytes harboring the MELAS mutation have severe deficiency in activities of Complex I and IV and a moderate deficiency of Complex II of the RC. The RC defects correlate with lower TEER values found in the EC cells with the mutation. Our results, though preliminary, suggest that the BBB is perturbed in the presence of the MELAS mutation. Further work with EC-astrocyte co-cultures aimed at directly testing paracellular permeability will provide insight into the integrity of the BBB in MELAS. This *in vitro* model of the BBB has the potential to unravel the complex pathogenic mechanism beyond molecular characterization of a devastating disease and to allow testing interventional strategies.

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Fig.1.
Repopulation of endothelial cells and astrocytes with the m.3243A>G mtDNA mutation:
scheme.

Fig.2. RFLP analysis of the MELAS mutation. (A) WT and MELAS endothelial cells and astrocytes. 1:Size marker, 2:Uncut, 3:MELAS cybrids, RN164, 4:hCMEC/D3-Clone 2 , 5:hCMEC/D3-Clone 10, 6:hCMEC/D3 WT 1, 7: hCMEC/D3 WT 2, 8:IHFA Clone 16, 9:IHFA Clone 21, 10: MELAS cybrids 11: IHFA WT and (B) WT and MELAS primary astrocytes. 1:MAG1, MELAS primary astrocytes, 2:WT primary astrocytes. WT cells show the 192 kb band; Mutant cells reveal the 97 and 72 kb diagnostic bands.

Fig.3.

Quantitation of mtDNA/nDNA ratios in the WT and MELAS EC and astrocyte clones. WT EC, (hCMEC/D3), MELAS EC clones, (hCMEC/D3-2 and hCMEC/D3-10), WT astrocytes (IHFA) and MELAS astrocyte clones, (IHFA-16 and IHFA-21). Outset: There is no significant change in the mtDNA/nDNA ratio in WT and MELAS cells during repopulation.

Fig.4. COX and SDH histochemistry of WT and MELAS EC and astrocytes. (A), WT and MELAS EC clones, (hCMECD3-2 and hCMECD3-10), and (B), WT and MELAS astrocyte clones, (IHFA-16 and IHFA-21). Staining for COX was reduced in the MELAS EC and astrocyte clones; SDH staining was reduced in both the MELAS EC clones and in one MELAS astrocyte clone.

Fig.5.

Mitochondrial translation and function in primary MELAS and WT astrocytes.

Immunostaining for mitochondrial COX subunit II is reduced in primary MELAS astrocytes, MAG1 (a) compared to normal (b), while the staining for nuclear encoded subunit PDH is normal in both (c, d) indicating defective translation of mitochondrial subunits. Histochemical staining for COX is low in the MELAS astrocytes (e) compared to normal (f); SDH histochemistry is normal in both (g, h).

Fig.6. TEER of WT (hCMEC/D3) and MELAS endothelial cell clones. Mutant clones, (hCMEC/D3 C1 10 and 2) had lower TEER readings compared to WT (hCMEC/D3) at every time point analyzed indicating increased permeability.

Biochemical analysis of respiratory chain enzymes in WT and MELAS endothelial cells and astrocytes.

CELLS	COX	SDH	P value	C I	P value	CS	P value	COX/ CS	SDH/ CS	CI / CS
Endothelial cells										
HCMEC/D3	124.6±4.8	27.48±5.8		256.58±16.32		78.2±7.3		1.59	0.35	3.28
HCMEC/D3 Clone 2	83.6±8.0	19.45±6.2	0.0022 *	12.34±5.2	0.0931	70.4±4.7	0.0022 *	1.18	0.28	0.17
HCMEC/D3 Clone 10	74.7±10.9	17.54±4.6	0.0022 *	98.55±8.1	0.0087	80.4±6.6	0.0022 *	0.93	0.22	1.23
Astrocytes										
IHFA WT	135.3±2.5	26.0±4.6		62.24±6.56		55.8±2.5		2.42	0.47	1.12
IHFA Clone 16	74.2±3.2	20.0±4.7	0.0022 *	13.59±3.37	0.0714	59.4±5.1	0.0022 *	1.25	0.34	0.23
IHFA Clone 21	70.5±3.8	25.2±3.7	0.0022 *	37.54±4.44	0.6991	54.2±3.7	0.0022 *	1.30	0.46	0.69

All activities: n moles/min/mg pm
* Significant at the 0.05 level of significance