Histopathological comparison of Kearns-Sayre syndrome and PGC-1α-deficient mice suggests a novel concept for vacuole formation in mitochondrial encephalopathy

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Abstract
Despite the current hypotheses about myelinic and astrocytic ion-dyshomeostasis underlying white (WM) and grey matter (GM) vacuolation in mitochondrial encephalopathies, there is a paucity of data on the exact mechanism of vacuole formation. To revisit the concepts of vacuole formation associated with mitochondrial dysfunction, we performed a comparative neuropathological analysis in Kearns-Sayre syndrome (KSS) and full-length peroxisome proliferator-activated receptor-γ coactivator-1α (FL-PGC-1α)-deficient mice, a recently proposed morphological model of mitochondrial encephalopathies. Brain tissues from an individual with genetically proven KSS (22-year-old man) and aged FL-PGC-1α-deficient and wild-type (male, 70-75-week-old) mice were analysed using ultrastructural and immunohistochemical methods, with a specific focus on myelin-related, oligodendroglial, axonal and astrocytic pathologies. Besides demonstrating remarkable similarities in the lesion profile of KSS and FL-PGC-1α-deficient mice, this study first provides morphological evidence for the identical origin of WM and GM vacuolation as well as for the presence of intracytoplasmic oligodendroglial vacuoles in mitochondriopathies. Based on these observations, the paper proposes a theoretical model for the development of focal myelin vacuolation as opposed to the original concepts of intramyelin oedema. Placing oligodendrocytes in the centre of tissue lesioning in conditions related to defects in mitochondria, our observations support the rationale for cytoprotective targeting of oligodendrocytes in mitochondrial encephalopathies, and may also have implications in brain aging and multiple sclerosis, as discussed.

Key words: Kearns-Sayre syndrome, PGC-1α, mitochondrial encephalopathy, vacuole, myelin, oligodendrocyte.

Introduction
Mitochondrial diseases comprise a group of inherited or sporadic neurological disorders in which proper mitochondrial function is compromised. Either the mitochondrial or nuclear genome is involved by the genetic alterations. Based on the symptomatic appearance, such diseases are divided into characteristic syndromes, including MELAS (mitochondrial encephalomyopathy, lactic acidosis, and stroke-like episodes) and Pearson syndrome (autosomal recessive, congenital, non-syndromic, encephalomyopathy).

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encephalomyopathy, lactic acidosis, and stroke-like episodes), MERRF (myoclonic epilepsy with ragged red fibres), LHON (Leber’s hereditary optic neuropathy), MNGIE (mitochondrial neurogastrointestinal encephalopathy), NARP (neuropathy, ataxia, retinitis pigmentosa), Leigh’s syndrome and Kearn-Sayre syndrome (KSS) [4,6,9,47], as well as the more recently characterized LBSL (leukoencephalopathy with brainstem and spinal cord involvement and lactate elevation) [39,48] and HBSL (hypomyelination with brainstem and spinal cord involvement and leg spasticity) [44]. The leading symptoms develop due to the progressive pathology in organs with high energy demand, resulting usually in early death. Though the distribution of neuropathological alterations is characteristic of a particular syndrome, all mitochondrial encephalopathies present in various degrees of vacuolation in the white (WM) and grey matter (GM), regional neurodegeneration with reactive astrogliosis and, less generally, capillary proliferation [4,6,9,47].

Recently, mice deficient in the expression of full-length peroxisome proliferator-activated receptor gamma coactivator-1α (PGC-1α) protein (FL-PGC-1α) have been suggested as a morphological model for mitochondrial diseases [43]. PGC-1α is a nuclear-encoded protein that plays important roles in the transcriptional regulation of mitochondrial function at several levels, including mitochondrial biogenesis, glucose and lipid metabolism as well as oxidative stress defence, and its dysfunction has been implicated in the pathogenesis of a number of neurodegenerative diseases both in humans and experimental animals [37,43]. FL-PGC-1α-deficient mice develop liver disease, decreased locomotion and muscle weakness [23], together with a spongiform leukoencephalopathy with wide-spread vacuolization accompanied by reactive astrogliosis in the brainstem and the cerebellar nuclei, resembling the neuropathological alterations seen in KSS [43].

According to the current notion, WM vacuoles in mitochondrial encephalopathies are linked to intramyelin ‘oedema’ due to preferential mitochondrial dysfunction in oligodendrocytes, presenting as intramyelin ‘bubbles’ due to splitting of the myelin sheath at the intraperiod line. In contrast, GM neuropil vacuoles are presumed to develop because of the failure of ATP-dependent ion transporters in astrocytic membranes [32,40,46]. While intraperiod line splitting is a common finding in cases with status spongiosus, the mechanism through which focal and circumscribed fluid accumulation develops in an extracellular compartment is only poorly understood. Thus, our knowledge about the origin of intramyelin and neuropil vacuolation remains speculative.

Our first study on FL-PGC-1α-deficient mice indicated that the majority of the observed vacuoles were associated with myelin in the absence of apparent axonal involvement [43]. Based on these observations, we aimed to evaluate the origin and nature of vacuole formation in aged FL-PGC-1α-deficient mice, in comparison with a human case of KSS. Our results emphasize the novel pathogenic role of the oligodendrocytes in the formation of both WM and GM vacuoles. Besides providing a better understanding of tissue lesioning in mitochondrial encephalopathies, our observations may also have implications for WM damage in multiple sclerosis as well as in the aging brain, related pathologies where mitochondrial dysfunction may also play key roles in the pathogenesis.

Material and methods

Patient and animals

Brain tissue of a male KSS patient with a 4.9 kbp common deletion in mtDNA died at 22 years of age was used for neuropathological analysis. The mother gave informed consent prior to the neuropathological work-up. The study was adhered to the tenets of the most recent revision of the Declaration of Helsinki.

For comparison, 70-75-week-old FL-PGC-1α-deficient and age-matched wild-type C57Bl/6J male mice were involved in this study. The animals were housed in cages (maximum 4 per cage) in standard conditions with 12-12 h light-dark cycle and ad libitum access to standard pellet food and water. The experiments were performed in accordance with the European Communities Council Directive (86/609/EEC) and were approved by the local Animal Care Committee.

Immunohistochemistry and histology

Formalin-fixed paraffin-embedded blocks of different regions of the KSS brain, including several neocortical areas, basal ganglia, thalamus, hippocampus, amygdala, brainstem and cerebellum, as well as a double hemispheric block at the levels of WM lesions were examined. The murine brains were removed on ice and halved at the midline immediately following decapitation. Half brains were fixed...
in 4% paraformaldehyde overnight and kept in 15% glycerol in 4°C until embedding in paraffin. The other halves of the murine brains were used for the frozen sections (i.e. for Oil Red O staining).

3-μm-thick sections were stained with Klüver-Barrera (Luxol and Fast red) and Oil Red O stainings. For immunohistochemistry we applied the following monoclonal antibodies (cross-reacting with mouse): anti-amyloid precursor protein (APP) (1 : 500, Millipore, Billerica, Mass., USA), phosphorylated and non-phosphorylated neurofilaments (clones SMI-31 and SMI-32, markers of axons and neuronal cell bodies; 1 : 5000 and 1 : 2000, respectively, Covance, Berkeley, CA, USA), TPPP/p25 (1 : 2000; a marker of mature oligodendrocytes [15]) and microtubule associated protein-2 (MAP-2, marker of neuronal cell bodies and dendrites; Millipore). Furthermore, the following polyclonal antibodies were used: antiglial fibrillary acidic protein (GFAP; 1 : 3000, Dako, Glostrup, Denmark), MBP (myelin basic protein; 1 : 400, Dako) and Iba1 (1 : 1000, Wako Chemicals, Osaka, Japan). The DAKO EnVision detection kit, peroxidase/DAB, rabbit/mouse (Dako) was used for visualization of antibody reactions. When applying mouse antibodies, we used the M.O.M. kit (Vector Laboratories, Burlingame, Calif., USA) to prevent the aspecific background staining of endogenous mouse immunoglobulins.

**Electron microscopy**

Human KSS samples from the basal ganglia (internal capsule) and WM lesions were immersion-fixed in 4% glutaraldehyde for 3 days. The animals used for electron microscopy were anaesthetised with isoflurane and were perfused transcardially with modified Hamori fixative (1.5% glutaraldehyde and 1% formaldehyde in phosphate buffer) for 18 min with a 10 ml/min flow, prior to decapitation and subsequent sample preparation.

The obtained central nervous system (CNS) samples were postfixed in 1% osmium tetroxide for 1-2 h, dehydrated through a series of graded ethanol and propylene oxide, and then embedded in Embed 812 resin (Electron Microscopy Sciences, EMS 14120). Semi-thin sections were stained with toluidine blue, blocks trimmed, and ultrathin sections stained with lead citrate and uranyl acetate. Specimens were examined using a JEM-100C transmission electron microscope.

**Results**

**Comparison of lesion profiles**

**Vacuolation**

Klüver-Barrera staining revealed a widespread spongiform change in both the KSS brain and FL-PGC-1α-deficient mice. In both, the vacuolation predominated in the WM; however, vacuoles in the GM neuropil were also observed with apparently lower frequency (Fig. 1A-F). The vacuolation commonly affected the internal capsule, striatal pencil fibres, cerebellar WM, thalamic fascicles, pyramidal tracts and, less intensively, the corpus callosum. Vacuoles were commonly present in the neuropil, most intensively in the brainstem, but also consistently present in the basal ganglia, thalamic nuclei and less intensively in the neocortex, where they showed a predilection toward the deep cortical layers. The severity of vacuolation was more prominent in the KSS brain than in experimental animals; in some regions with coarse cystic-necrotic lesions and myelin pallor, while in some others with demyelinated foci (e.g. postcentral region, cerebellar WM, some of the pencil fibres) (Fig. 1C and F). Demyelination and cystic-necrotic lesions were undetected in FL-PGC-1α-deficient mice. Notably, the examined aged wild-type brains presented vacuoles showing similar appearance and distribution as their FL-PGC-1α-deficient counterparts; however, their frequency was remarkably lower in all examined regions (Fig. 1A-B, D-E).

**Astrogliosis**

GFAP staining revealed moderate to severe reactive astrogliosis in the brainstem and cerebellum of both the KSS and the FL-PGC-1α-deficient brains (Fig. 1G-I). In the KSS brain, astrogial reaction was observed in the tectal midbrain, the area of the inferior olive in the medulla oblongata, the dentate nucleus of the cerebellum and the Purkinje cell layer (Bergmann gliosis). In the FL-PGC-1α-deficient mice, severe reaction was present in the medulla oblongata and the pons often in a confluent pattern involving large areas without being limited to certain groups of nuclei, whereas mild reaction with patchy astrogliosis was observed in the midbrain and in the deep cerebellar nuclei. Notably, the caudate-putamen of PGC-1α-deficient mice were free of astrogial reaction even at this age (Fig. 2), supporting our prior observations [43].
Axonal pathology

The intensity of axonal destruction in the KSS brain was variable and generally followed that of the vacuolar change. Only the severely affected cystic-necrotic lesions showed axonal loss or swelling, whereas most of the moderately vacculated areas were devoid of axonal pathology (Fig. 3C), except for scattered swellings and APP-positive spheroids indicating acute-subacute impairment of axonal transport (Fig. 3F). Regions with severe axonal involvement were accompanied by reactive microgliosis (Fig. 3I). The axons in the FL-PGC-1α-deficient mice were generally well preserved, and the patterns of APP, neurofilament and microglia stainings were similar to that observed in wild-type (Fig. 3A-B, D-E and G-H).

Characterization of vacuoles

White matter vacuoles

Vacuoles within the WM were surrounded by rings of MBP-positive myelin in both the KSS and FL-PGC-1α-deficient brains (Fig. 4A). The vacuoles...
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Neuropil vacuoles

Staining for neuronal (MAP-2 and SMI-32) or astrocytic (GFAP) antigens sparsely revealed associations of neuropil vacuoles with these cell-types in either the KSS or the FL-PGC-1α-deficient brains (Fig. 7). Staining for MBR however, unveiled that the vast majority of neuropil vacuoles were clearly encompassed by a myelin-positive rim, suggesting the same intramyelinic localization as for vacuoles within the WM (Fig. 4B). Neuropil vacuoles were also frequently associated with oligodendrocytes in sections immunostained for TPPP/p25 (Fig. 4C). Such close contacts of oligodendrocytes and vacuoles were also observed by electron microscopy. Furthermore, vacuoles (sometimes multiloculated) could frequently be identified within the cytoplasm of glial cells, which, due to the lack of glial fibres observed in the cytoplasm, also appeared to be oligodendrocytic (Fig. 5C). Likewise myelin ‘bubbles’, these membrane-bound vacuoles often contained myelin-like figures, and they occasionally coalesced occupying most of the oligodendroglial cytoplasm and lead to the swelling of the cells (Fig. 5C). These observations were seen in both the KSS and the FL-PGC-1α-deficient brains.

Discussion

In addition to its central role in mitochondrial encephalopathies, mitochondrial dysfunction has been associated with various neurodegenerative CNS disorders [17], and is supposed to play important roles in the pathogenesis of WM lesioning in multiple sclerosis (MS) [7] as well in the aging brain [38]. Though the exact mechanism of vacuole formation in mitochondrial encephalopathies is not yet revealed, intramyelin WM vacuoles are presumed to develop due to intramyelinic oedema secondary to mitochondrial dysfunction of oligodendrocytes, manifesting in splitting of the myelin sheath at the intraperiod line [32,40,46]. On the other hand, GM neuropil vacuoles are presumed to develop due to ion transport disorder of astrocytic membranes [32,40,46].

Revisiting the above concepts of vacuole formation in conditions associated with mitochondrial...
dysfunction, our study provided two main novelties. A major finding of our study is that it demonstrates commonalities of vacuolation in the WM and GM, placing oligodendrocytes in the centre of disease pathogenesis. Indeed, our observation that the vast majority of GM neuropil vacuoles are clearly myelin-bound in both the KSS and FL-PGC-1α-deficient brains indicates that the cellular localization and thus the mechanism of vacuole-formation is most likely similar, irrespective of whether they are in WM or GM. This observation can explain the phenomenon widely observed in mitochondrial encephalopathies – including PGC-1α-deficient mice – that cortical vacuoles show a predilection toward the deeper layers of the neocortex, as the density of myelinated fibres apparently gradually decreases by approaching the superficial layers. Importantly, this could also explain the predilection of GM neuropil vacuoles toward the reticular area of the brainstem, thalamus, deep cerebellar nuclei, and basal ganglia adjacent to the internal capsule, as these GM regions are intermingled with the WM. Interestingly, these regions are

Fig. 3. Axonal pathology. Axons in areas with moderate vacuolation in KSS were relatively intact with sparse presence of axonal swellings (C; SMI-31). Such areas also showed scattered appearance of APP+ axonal spheroids indicative of subacute axonal transport impairment in KSS (F; APP). Regions with cystic-necrotic lesions showed robust microglia accumulation in KSS (I; Iba). These pathologies were virtually absent in the FL-PGC-1α-deficient mice and the immunohistochemical patterns were rather similar to that in aged wild-types (A-B, D-E and G-H). Note the well-preserved axons dodging between multiple vacuoles (B), similarly to that seen in KSS (C).
Fig. 4. Immunohistochemical characterization of vacuoles. Vacuoles within the WM are surrounded by rings of myelin (A; MBP). Likewise WM myelin ‘bubbles’, vacuoles within the GM neuropil are also encompassed by a myelin-positive rim, suggesting an identical origin (B; MBP). Oligodendroglial cells in close contact with and/or localized within the inner edge of either single or multiloculated vacuoles can frequently be detected by immunohistochemistry (C; TPPP/p25), suggesting an important role of oligodendrocytes in vacuole formation. Note some of the larger vacuoles being separated to multiple chambers by oligodendroglial processes (C). Note also the glial cell encompassing a vacuole with its MBP+ process (indicated by arrows, A – left, B – right).
Fig. 5. Ultrastructural characterization of vacuoles. Intramyelin vacuolation can be identified also by electron microscopy (longitudinal section, A; transverse section, B). In accordance with immunohistochemical findings, vacuoles, often multiloculated, can frequently be identified within the cytoplasm of oligodendroglial cells by electron microscopy (C).

the predilection areas for the development of GM vacuolation in hepatic encephalopathy as well [52]. Considering this overlapping predilection of spongy change between hepatic and mitochondrial encephalopathies, and that mitochondrial disorders, including PGC-1α deficiency [23], also present with hepatic involvement, the contribution of liver insufficiency to the development of mitochondrial status spongio-
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Fig. 6. Distinct types of intramyelin vacuoles in Kearns-Sayre syndrome (KSS). Asterisk denotes a huge ‘classical’ intramyelin vacuole originating from splitting at the intraperiod line. Arrows indicate the multiple presence of adaxonal vacuoles, some of which appear to originate from splitting between the two innermost myelin lamellae at higher power. The frequent appearance of adaxonal vacuoles may underpin the role of intra-oligodendroglial vacuolar change in the development of intramyelin vacuoles in mitochondrial encephalopathies such as KSS.

sus cannot be excluded either. Notably, intramyelin vacuolation has been frequently described in other experimental, veterinary and human metabolic conditions (Table I), suggesting that this change might be a general response to various insults that compromise the metabolism of the myelin sheath and/or oligodendrocytes.

As a second novelty, the ultrastructural analysis revealed the common appearance of intracellular, often multiloculated formation of vacuoles within oligodendroglial cells both in the KSS case and the FL-PGC-1α-deficient mice. This finding was supported by the immunohistochemical observation of TPPP/p25-positive oligodendrocytes directly attaching to and sometimes bulging into vacuoles within the neuropil. The finding, however, that some vacuoles were observed closely attached to and partly encompassed by neuronal structures in MAP-2 and SMI-32 stainings suggests that at least a small proportion of vacuoles within the neuropil can be of neuronal origin, and that the mechanisms which lead to excessive intracellular membrane-bound fluid accumulation may affect different cell-types within the CNS, though to different extents. This hypothesis is supported by the report on CNS vacuoles observed also in neuron-specific PGC-1α knockout mice [25].

Interestingly, though intracellular oligodendroglial vacuoles have not been previously described in mitochondrial diseases, their presence is not unprecedented in pathologies associated with mitochondrial dysfunction and/or severe cellular stress. Indeed, chronic feeding of mice with cuprizone, a copper-chelating mitochondrial toxin associated with megamitochondria [41] and alterations in complex IV and superoxide dismutase (SOD) activity [1], evokes a CNS pathology comprising vacuole formation in the pons, midbrain, thalamus, cerebral and cerebellar WM, as well as in deep cortical layers [42]. It was described that cuprizone-induced intramyelin vacuolation was due to splitting at the intraperiod line and were not stained by Sudan IV (equivalent with Oil Red O in our study) [42]. Additionally, the authors reported the presence of oligodendrocytes with enlarged cytoplasm containing multiloculated vesicles, and that some of these cells were juxtaposed to myelin sheaths, where a thin myelin layer appeared to form the glial membrane [42]. These findings are strikingly similar to those observed in our study. A further similarity between cuprizone-induced and mitochondrial status spongiosus is that cuprizone toxicity in doses evoking demyelination associates with oligodendroglial apoptosis [1], a phenomenon recently described in KSS [22]. Our observation of intra-oligodendroglial vacuolation in mitochondrial encephalopathy expands the spectrum of disorders where this has been described (Table II).

Though the neuropathological alterations in MS are results of a complex aetiology including autoimmunity, demyelination, neuronal and axonal degeneration, the central role of oligodendrocytes has also been suggested [15], which gives our observations a wider implication. The interrelation between mitochondrial leukoencephalopathies and MS is well exemplified by the fact that the above mentioned cuprizone intoxication, presenting with a highly similar neuropathology to that observed in KSS and FL-PGC-1α-deficient mice in our study, is in fact a widely applied toxin model of MS [1,27]. It has also been suggested that though a moderate mitochondrial dysfunction alone may not cause selective demyelination directly, its effect to evoke a disintegrated myelin structure may expose the sheath to
further damage, e.g. by complex immune-mediated mechanisms [20]. Indeed, mtDNA mutations have been proposed to affect the CNS on a common metabolic basis, which may occasionally aggravate or initiate autoimmune pathology leading to MS-like lesions [20].

Besides demyelinating WM disease, our study also has implications for understanding WM lesions
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in the aging brain, a frequent pathology which includes the formation of intramyelin ‘balloons’ due to intraperiod line splitting [34], similar to that seen in mitochondrial disorders. Our observation that aged wild-type mice also develop vacuoles to an apparently slighter extent but at the same predilection areas as their FL-PGC-1α-deficient counterparts recapitulates previous observations [10]. Based on these, we propose that vacuole formation in mitochondrial encephalopathies and their representative animal models (e.g. PGC-1α-deficient mice) might also be regarded as an accelerated form of ‘normal’ WM degeneration, which underpins the role of (oligodendroglial) mitochondrial dysfunction in aging.

Although the potential role of oligodendrocytes in WM vacuolation in mitochondrial encephalopathies has already been suggested, the fundamental concepts included (1) a disrupted ion-homeostasis of the sheath, (2) a dysfunction of the blood-brain barrier, in both cases with consequent development of ‘intramyelin oedema’ [32,40,46]. These hypotheses, however, do not explain why vacuoles develop focally and how multiple vacuoles can be found within the same internode, instead of a complete splitting and diffuse loosening of the sheath between all lamellae. We propose that chronic mitochondrial dysfunction in a yet unknown pathway leads to the formation of multiple intra-oligodendroglial fluid-filled vacuoles. Increased intracellular content might provoke splitting between the intracellular surfaces of the myelin sheath (major dense line). Due to their firm connections at the macromolecular level, this would cause tears and focal myelin disruptions, allowing the vacuolar content to access into the virtual space between the loosely attached extracellular surfaces (intraperiod lines). Consequently, this could evoke the formation of focal splits and eventually myelin bubbles. Accordingly, disruption of the lateral loops would result in intraperiod line splitting at the corresponding levels, whereas leakage from the inner tongue would cause adaxonal swelling between the axolemma and the innermost myelin lamellae or between the two innermost layers. Supporting
this theoretical consideration, such distinct types of intramyelin vacuoles have indeed been described in experimental status spongiosus [36,50], and could also be observed in our study (Fig. 6).

Conclusions
In addition to a detailed comparative neuro-pathological characterization of a recently proposed murine model of mitochondrial encephalopathy and human KSS, this study first provides morphological evidence for the identical intramyelinic nature of WM and GM vacuolation as well as for the presence of intra-oligodendroglial vacuoles in mitochondrial disease, which may have a pathogenetic role in the development of intramyelin vacuoles, providing a possible source of focal myelin splitting. Our observations place oligodendrocytes in the centre of the pathogenesis of CNS lesioning in association with chronic mitochondrial dysfunction both in WM and GM, which is in line with the recognition that oligodendrocytes, contrasting astrocytes [11], are most sensitive to mitochondrial stress, exceeding the vulnerability of neurons [8]. This may serve as rationale for cytoprotective targeting of the oligodendrocytes in mitochondrial encephalopathies as well as in other disorders with vacuole formation and myelin degeneration.

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Disclosure
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References
18. Kanno T, Sasaki S, Yamada N, Kawasaki K, Tsuchitani M. Hexachlo-
20. Levente Szalardy, Mate Molnar, Rita Torok, Denes Zadori, Laszlo Vecsei, Peter Klivenyi, Paweł Piotr Liberski, Gabor Geza Kovacs