Molecular Genetics and Metabolism xxx (xxxx) xxx–xxx

Contents lists available at ScienceDirect

Molecular Genetics and Metabolism

journal homepage: www.elsevier.com/locate/ymgme

Erratum

New *in vitro* model derived from brain-specific *Mut-/-* mice confirms cerebral ammonium accumulation in methylmalonic aciduria

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ARTICLE INFO

Keywords:
Methylmalonic aciduria
Methylmalonate
2-Methylcitrate
Neurotoxicity
Hyperammonemia
Neuroinflammation

ABSTRACT

Background: Methylmalonic aciduria (MMAuria) is an inborn error of metabolism leading to neurological deterioration. In this study, we used 3D organotypic brain cell cultures derived from embryos of a brain-specific $Mut^{-/-}$ (brain KO) mouse to investigate mechanisms leading to brain damage. We challenged our *in vitro* model by a catabolic stress (temperature shift).

Results: Typical metabolites for MMAuria as well as a massive NH4$^+$ increase were found in the media of brain KO cultures. We investigated different pathways of intracerebral NH_4^+ production and found increased expression of glutaminase 2 and diminished expression of GDH1 in $Mut^{-/-}$ aggregates. While all brain cell types appeared affected in their morphological development in $Mut^{-/-}$ aggregates, the most pronounced effects were observed on astrocytes showing swollen fibers and cell bodies. Inhibited axonal elongation and delayed myelination of oligodendrocytes were also noted. Most effects were even more pronounced after 48 h at 39 °C. Microglia activation and an increased apoptosis rate suggested degeneration of $Mut^{-/-}$ brain cells. NH_4^+ accumulation might be the trigger for all observed alterations. We also found a generalized increase of chemokine concentrations in $Mut^{-/-}$ culture media at an early developmental stage followed by a decrease at a later stage.

Conclusion: We proved for the first time that $Mut^{-/-}$ brain cells are indeed able to produce the characteristic metabolites of MMAuria. We confirmed significant NH_4^+ accumulation in culture media of $Mut^{-/-}$ aggregates, suggesting that intracellular NH_4^+ concentrations might even be higher, gave first clues on the mechanisms leading to NH_4^+ accumulation in $Mut^{-/-}$ brain cells, and showed the involvement of neuroinflammatory processes in the neuropathophysiology of MMAuria.

1. Introduction

Methylmalonic aciduria (MMAuria; MIM #251000) is an inborn error of metabolism characterized by accumulation of methylmalonate (MMA), propionate and 2-methylcitrate (2-MCA) in tissues and body fluids. These accumulations result from defects in the activity of methylmalonyl-CoA mutase (MUT, EC 5.4.99.2). MMA is the major accumulating metabolite in this disease [1]. A synergistic toxic effect of MMA, PA and 2-MCA on brain cells has been suggested [2].

Clinical symptoms are ketoacidosis, lethargy and failure to thrive, often leading to coma or even death in the newborn period. In surviving patients, organ-specific complications occur mainly in brain and kidney [3, 4].

Current treatment approaches consist mainly in low-protein diet,

Abbreviations: 2-MCA, 2-methylcitrate; AKG, α-ketoglutarate; BBB, blood-brain barrier; CSF, cerebrospinal fluid; CNS, central nervous system; cTIN, chronic tubulointerstitial nephritis; DIV, day *in vitro*; FGF, fibroblast growth factor; GalC, galactocerebroside; GDH, glutamate dehydrogenase; GFAP, glial fibrillary acidic protein; Gln, Glutamine; Glu, Glutamate; Gly, glycine; hTEC, human tubular epithelial cells; Iba1, Ionized calcium binding adaptor molecule 1; KO, knock-out; LC/MS-MS, liquid chromatography coupled to tandem-mass spectrometry; MBP, myelin basic protein; MUT, methylmalonyl-CoA mutase; MMA, methylmalonate; MMAuria, methylmalonic aciduria; NGF, nerve growth factor; NT-3, neurotrophin-3; NH_4^+, ammonium; PBS, phosphate-buffered saline; p-NFM, phosphorylated medium weight neurofilament; ROS, reactive oxygen species; THF, Tetrahydrofolate; WT, wild-type

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https://doi.org/10.1016/j.ymgme.2018.06.008
Received 14 March 2018; Received in revised form 14 June 2018; Accepted 14 June 2018

carnitine and cobalamin supplementation. High-caloric emergency treatment intends to prevent or reverse the catabolic state. Liver or kidney transplantation has been used as a therapeutic strategy to correct the enzymatic defect [5]. However, since neurons express the catabolic pathway of propionate metabolism, the enzymatic defect is only corrected in the periphery after organ transplantation [6]. Neurological deterioration, as cerebral atrophy, delayed myelination and extrapyramidal syndromes have been observed in MMAuria patients after liver transplantation [6, 7]. Early diagnosis and current treatment strategies are only partially effective in preventing neurological damage. These neurological changes are very complex as intellectual disability may or may not be present even in those with severe disease [8].

The pathophysiological mechanisms underlying neurologic deterioration in MMAuria patients are still poorly understood.

Previous results on 3D organotypic brain cell cultures in aggregates derived from wild type rat embryos and challenged with 2-MCA showed alterations of the morphology of developing brain cells, increased ammonium (NH_4^+) concentrations and cell death by apoptosis [9, 10].

Excessive intracerebral NH_4^+ production might be a key mechanism in the pathophysiology of brain damage in MMAuria. Several enzymatic pathways can generate NH_4^+ in the mammalian brain [11]. The most important ones are (1) glutamate dehydrogenase (GDH), (2) the purine nucleotide cycle and (3) glutaminase, in which glutamate, aspartate and glutamine are the source of NH_4^+ generation (Fig. 1). GDH is highly expressed in brain and produces NH_4^+ by catalyzing the reversible oxidative deamination of glutamate. Glutamate dehydrogenase 1 and 2 genes (*GLUD1* and *GLUD2*) encode two GDH isoenzymes named GDH1 and GDH2, which show a high sequence homology. Both are expressed in brain. GDH2 expression is known to be restricted to astrocytes of the cerebral cortex [12, 13], while GDH1 is widely expressed in the brain [14]. Glutaminase is considered to be a major source of cerebral NH_4^+ production [15]. This enzyme degrades glutamine (Gln) in the CNS by hydrolytic deamination leading to glutamate (Glu) and NH_4^+ [16]. Several isoforms of glutaminases have been described in CNS, both neuronal and glial [16].

In 2003, Peters et al. developed a knockout (KO) mouse model for MMAuria [17]. Homozygous KO mice ($Mut^{-/-}$) appear normal at birth but die at 24 h of age. Their biochemical phenotype is similar to that in MMAuria patients. Several other models have been developed in the meantime [18–22].

For this study we used a brain-specific $Mut^{-/-}$ (brain KO) mouse model that recapitulates the key biochemical features of MMAuria. The use of this brain KO mouse allowed the generation of a new 3D *in vitro* model for brain damage in MMAuria that is closer to the *in vivo* conditions. In this article, we describe the characteristics of our new *in vitro* model for MMAuria and the involved pathophysiologic mechanisms.

2. Materials and methods

2.1. Generation of a brain-specific $Mut^{-/-}$ (brain KO) mouse

For the generation of *Mut*-floxed mice, stem cell targeting was used in collaboration with Polygene (Rümlang, Switzerland). C57Bl/6 N-derived ES cells were electroporated with the linearized EUCOMM vector (EUCOMM vector ID 47381), where exon 3 of the *Mut* gene was flanked by loxP sites; additionally, an FRT-flanked targeting cassette was inserted into intron 2, which included a neomycin resistance gene and a lacZ reporter (Fig. 2A). ES cell clones, positively verified for homologous recombination, were injected into blastocysts of C57Bl/6, resulting in chimeric mice which were set up for breeding with Flp-deleter mice. The offspring was screened by PCR and sequenced for i) the presence of the remaining FRT-site in the targeted allele to identify germ line transmitters; ii) the absence of the neomycin cassette; iii) the distal loxP site; and iv) the Flp transgene. Mice were bred to homozygosity for the floxed *Mut* allele. Subsequently, $Mut^{flox/flox}$ mice were crossed with Nestin-Cre (Nes-Cre) mice (RRID:IMSR_JAX:003771), which express Cre recombinase driven by the Nestin promotor [23], in order to generate brain-specific $Mut^{-/-}$ ($Mut^{flox/flox;Nes\text{-}Cre}$, brain KO) mice. Genotyping was performed on genomic DNA from ear punch biopsies using the primers 5′-CTATGAACGGAGCTGTCATC-3′ (forward) and 5′-GGAGGTGGCAAACACATAAG-3′ (reverse) for the flox allele and 5′-GCACTGATTTCGACCAGGTT-3′ (forward) and 5′-CCCGGCAAAACA GGTAGTTA-3′ (reverse) for the Nes-Cre allele using beta actin as a positive control with the primers 5′-TGTTACCAACTGGGACGACA-3′ (forward) and 5'-GACATGCAAGGAGTGCAAGA-3′ (reverse).

2.2. Quantitative real time PCR analysis

Total RNA (RNAeasy Mini kit, QIAGEN) was extracted from either frozen mouse tissue lysates or aggregates and analyzed for expression using specific probes for *Mut* (Mm00485312_m1) and *Actb* (Mm02619580_g1) *via* the Taqman Gene Expression Master Mix on a 7900HT Fast Real-Time PCR System.

2.3. 3D organotypic cultures of developing brain cells

3D organotypic brain cell aggregates are highly organized cultures

(1) $Glu + NAD^+ + H_2O \rightleftharpoons AKG + NADH + NH4^+$

(2) $AMP + H_2O \rightarrow IMP + NH4^+$

(3) $Gln + H_2O \rightarrow Glu + NH4^+$

(4) $AKG + Ala \rightleftharpoons Glu + Pyruvate + NH4^+$

(5) $Gly + NAD^+ + H_2O + THF \rightarrow NADH + H^+ + HCO_{3-} + NH4^+ + 5\text{-}10\text{-}methyleneTHF$

Fig. 1. Pathways of endogenous intracerebral $NH4^+$ production. (1) Glutamate dehydrogenase reaction, (2) AMP deaminase reaction, (3) Glutaminase reaction, (4) Alanine transaminase reaction, (5) Glycine cleavage system reaction.