Impairment of methyl cycle affects mitochondrial methyl availability and glutathione level in Down's syndrome

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1. Introduction

Down's syndrome (DS) is the most common autosomal trisomy among live births. It is caused by trisomy of either the entire or critical portions of human chromosome 21. The origin of the extra chromosome 21 has been shown to be maternal in about 93% of the cases and about 7% of the cases are due to paternal non-disjunction followed by a very small proportion of the cases that are due to postzygotic mitotic non-disjunction [1]. It has therefore been suggested that the risk of DS is closely related to maternal age and most likely linked to meiotic error caused by the aging of oocytes. The clinical presentation of DS is complex and variable. The most striking features are associated with an increased incidence of congenital abnormalities, neurodevelopmental impairment and accelerated aging [2].

Although the perception of DS as a metabolic disease is not prevalent, overexpression of genes encoding specific enzymes directly leads to biochemical aberrations that affect multiple interacting metabolic pathways, culminate in cellular dysfunction and contribute to the pathogenesis of DS. The one-carbon metabolism pathway is greatly affected by overexpression of cystathionine-beta-synthase (CBS), located on chromosome 21, which modifies the levels of several intermediates of the cellular one-carbon metabolism in DS, among them the methyl donor S-adenosylmethionine (SAM). These deviations are likely to impact mitochondrial status, which is already defective in DS pathogenesis [9]. Most studies performed on DS cell cultures and tissues have shown reduced mitochondrial redox activity and membrane potential [10], ATP depletion [11], changes in expression of genes involved in the Krebs cycle and oxidative

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Abstract

In Down's syndrome there is evidence that increased gene expression coding for specific cystathionine-beta-synthase (CBS), located on chromosome 21, which modifies the levels of several intermediates of the cellular one-carbon metabolism in DS, among them the methyl donor S-adenosylmethionine (SAM). These deviations are likely to impact mitochondrial status, which is already defective in DS pathogenesis [9]. Most studies performed on DS cell cultures and tissues have shown reduced mitochondrial redox activity and membrane potential [10], ATP depletion [11], changes in expression of genes involved in the Krebs cycle and oxidative
phosphorylation [7,8,12] and reduced activity of mitochondrial enzymes [8]. Moreover, impaired mitochondrial DNA (mtDNA) repair after oxidative damage [12–14] and mtDNA defects have been observed. Recently, the activity of ATP synthase, ADP/ATP carrier (AAC) and adenylate kinase has been reported to be lower in human skin fibroblasts from DS compared with euploid fibroblasts [15].

Methylation processes are known to occur in mitochondria and rely on the transport of cytosolic SAM by the SAM carrier (SAMC) [16]. Any event lowering cytosolic SAM availability is destined to affect mitochondrial methylation which is known to exist but studies of the possible methylation targets are absent. All these data together suggest the interesting possibility that the abnormal one-carbon metabolism could play a role in affecting the status of DS mitochondria and eventually in the pathogenesis of the disease. By quantifying several intracellular metabolites related to one-carbon metabolism in DS cells compared to control cells we demonstrate that indeed mitochondria are affected by cytosolic methyl imbalance. We also report some preliminary findings on a possible target for mitochondrial methylation that is modified in DS compared to control cells.

2. Materials and methods

2.1. Cell growth and fractionation

Epstein-Barr virus-immortalized lymphoblastoid cells of six children ranging from 1 to 13 years old with DS and of six age-matched controls were grown in Roswell Park Memorial Institute (RPMI) 1640 medium with 20% fetal bovine serum supplemented with 2 mM-glutamine, 100 U penicillin, 160 mg/l gentamicin sulfate and 100 μg/ml streptomycin at 37 °C in 5% CO₂.

For cell fractionation 2×10⁶ cells of each sample were lysed within a Dounce tissue grinder in the presence of the lysis buffers provided by the Mitochondria Isolation Kit for Cultured Cells (Pierce), then centrifuged at 700×g for 10 min at 4 °C. Mitochondria were pelleted by centrifuging the supernatant at 15,000×g for 15 min and used for quantification of intramitochondrial metabolites. The obtained post-mitochondrial supernatant was used for quantification of cytosolic metabolites. For both cellular fractions protein content was determined by using the Bradford protein assay.

2.2. Quantification of cytosolic metabolites by mass spectrometry

Sample volumes corresponding to 1 mg proteins of the post-mitochondrial supernatants were extracted with phenol/chloroform 1:1. A Quattro Premier mass spectrometer interfaced with an Acquity UPLC system (Waters) was used for ESI-LC/MS/MS analysis. Standards preparation and calibration curves were prepared according to Piraud and coworkers [17]. The multiple reaction monitoring transitions monitored in the positive ion mode were m/z 399.15–250.10 for SAM, m/z 121.93–76.06 for cysteine, m/z 148.20–84.09 for glutamate, m/z 149.87–56.32 for methionine, m/z 308.28–179.00 for GSH, and m/z 613.20–484.16 for GSSG. Results were calculated using QuanLynx on a Windows XP operating system.

2.3. Quantification of mitochondrial metabolites by mass spectrometry

Sample volumes corresponding to 0.5 mg of mitochondrial proteins were extracted with phenol/chloroform 1:1. The multiple reaction monitoring transitions monitored in the positive ion mode for SAM, GSH and GSSG and amino acids by following the transitions and chromatographic procedure indicated in 2.2.

2.4. Real-time PCR

Total RNA was extracted from 1×10⁶ lymphoblastoid cells and transcribed by reverse transcriptase. Then real-time PCR was carried out as reported [18]. Assay-on-demand for human SAMC (cat. No. Hs00384779_m1) and human β-actin (cat. No. Hs99999903_m1) were purchased from Applied Biosystems. The transcript levels were normalized against the expression levels of β-actin.

2.5. Isolation of nuclear and mitochondrial DNA

For genomic DNA isolation 2×10⁶ cells of each sample were lysed within a Dounce tissue grinder, then centrifuged at 700×g for 10 min at 4 °C. The pellet was used for nuclear DNA extraction by following GenElute™ Mammalian Genomic DNA Miniprep Kit protocol. Mitochondrial DNA was purified with the mtDNA Isolation Kit (Biovision, Mountain View, USA) from 5×10⁶ cells for each sample. Extracted DNA samples were checked for mtDNA integrity and purity by electrophoresis on a 0.7% agarose gel. Absence of nuclear DNA was also tested by attempting to amplify specific genomic DNA regions. For each DNA sample, mitochondrial specificity was tested by PCR amplification of several mitochondrial target sequences as described in Dani et al. [19].

2.6. Quantification of mtDNA methylation

Quantification of the level of mtDNA methylation was obtained by following the method described by Song et al. [20]. Briefly, mitochondrial DNA (about 2 μg) was digested by nuclease S1 (800 U) at 45 °C for two hours, phosphodiesterase I (0.04 U) at 37 °C for two hours, alkaline phosphatase (3 U) at 37 °C for one hour. For mass spectrometry analysis of 5-methyl-2′-deoxycytidine (mdC), 2′-deoxycytidine (dc), using 2′-deoxyguanosine (dg) as internal standard as described [20], the following multiple reaction monitoring transitions were identified as follows: m/z 242.10–126.10 for mdC, m/z 228.10–112.10 for dc and m/z 268.1–152.3 for dc. Results were calculated using QuanLynx on a Windows XP operating system.

3. Results

3.1. Abnormal cytosolic levels of one-carbon metabolites in DS cells

In order to evaluate changes in the one-carbon metabolism, we quantified some substrates by LC-MS/MS analysis involved in the methyl metabolism in the cytosol and of Epstein–Barr virus-immortalized lymphoblastoid cells from both DS and control subjects. The validated method to quantify amino acids and derivatives

<table>
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<tr>
<th>Table 1</th>
<th>Cytosolic metabolite levels of DS and control cells.</th>
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<tr>
<td>Score</td>
<td>Met⁴, Cys⁵, Glu⁶, Total GS⁷, GSSG/GSH, SAM⁸, Ser⁹, Age</td>
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<tr>
<td>DS patients (N = 6)</td>
<td>25.95±6.23, 6.80±0.95, 0.52±0.09, 46.03±3.12, 0.91±0.30, 0.48±0.10, 32.21±11.80, 5.3±4.0</td>
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⁴ Met, methionine; Cys, cysteine; Glu, glutamate; Total GS, total glutathione; GSSG, glutathione in the oxidized form; GSH, glutathione in the reduced form; SAM, S-adenosylmethionine; and Ser, serine.

⁵ Data are expressed in mmol/mg cytosolic proteins.

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⁷ P<0.05.

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described by Piraud [17] was followed. As shown in Table 1, cytosolic SAM level was reduced in DS subjects respect to controls. Methionine, which is the precursor of SAM, was also reduced. Since the methyl metabolism replenishes glutathione in the reduced form, we measured the levels of metabolites involved in the glutathione synthesis. The levels of cysteine as well as glutamate increased in DS as compared to control cells (Table 1). These results were accompanied by a decreased total glutathione level in DS compared to control cells and an abnormal accumulation of the oxidized versus the reduced form of glutathione. By contrast, serine did not change between control and DS cells. These data suggest that the one-carbon metabolism is abnormal in DS compared to control cells and this result may impact mitochondrial methyl status as well.

3.2. Overexpression of the mitochondrial SAM carrier in DS cells

On the basis of the quantification observed for the one-carbon metabolites, we investigated whether the different levels of these cytosolic substrates (particularly SAM) could be linked to a different expression levels in DS versus control cells of the mitochondrial carrier that transports SAM into mitochondria [16] and thereby allowing methylation reactions in this subcellular compartment. We assessed the level of SAMC expression in both DS and control cells. Surprisingly, our real-time PCR experiments performed on mRNA extracted from lymphoblastoid cells revealed that expression of SAMC is at least 50% higher in DS respect to controls (Fig. 1).

3.3. Decreased SAM availability in DS mitochondria

We then evaluated changes in one-carbon metabolism in mitochondria of Epstein–Barr virus-immortalized lymphoblastoid cells from both DS and control subjects. Mitochondria were extracted and submitted to LC-MS/MS analysis. As shown in Table 2, mitochondrial SAM level was reduced in DS versus control cells. In addition, total glutathione was lower and the GSSG/GSH ratio could not be quantified due to the extremely low levels of GSH in DS compared to control cells.

To ascertain whether the lower mitochondrial availability of SAM of DS cells affects methylation, we chose to investigate mtDNA as possible target of methylation. Purified DNA samples from Epstein–Barr virus-immortalized lymphoblastoid cells were digested and analyzed by mass spectrometry following by the validated method described by Song and co-workers [20]. Fig. 2 shows the chromatogram for the monitored transitions. Methylation of DNA was expressed as ratio [mdC]/([mdC]+[dC]) (Table 2). We confirm the increase in nuclear DNA methylation in DS compared to control cells (data not shown), as also reported by Pogribna et al. [21]. To assess the level of mtDNA methylation we carried out several tests to exclude any possible contamination with genomic DNA. Gel electrophoresis of the isolated mtDNA samples shown in all cases a single band at about 15 kb (Fig. 3A). Absence of nuclear DNA was also tested by amplifying several specific nuclear DNA regions from the isolated mtDNA samples. None of these attempts provided amplification products (data not shown). Furthermore, mitochondrial specificity of the isolated DNA samples was tested by PCR amplification of several mitochondrial target sequences: Fig. 3B shows a specific fragment of about 500 bp, corresponding to the region from 14,018 bp to 14,508 bp of the mitochondrial genome. Our results demonstrated that mtDNA was hypomethylated in DS compared to the corresponding age-matched controls (Table 2).

4. Discussion

In the present study we have for the first time analyzed the consequences of the abnormal cytosolic methyl status on the mitochondrial methyl availability by quantifying SAM, glutathione and a possible target of methylation, mtDNA in mitochondria from DS subjects. In the context of methylation processes, SAM is required as a methyl donor for different methylation reactions not only in the cytosol, but also in mitochondria. SAM is transported into mitochondria by the SAM mitochondrial carrier (SAMC), which is encoded by SLC25A26 gene, in exchange for S-adenosylhomocysteine [16]. The presence of SAM is necessary for mitochondrial function prompted us to investigate the methylation status in mitochondria from DS cells, in which the cellular methylation capability is impaired. The decreased availability of SAM in DS cells, that we confirmed, limits the mitochondrial uptake of the methylating agent resulting in reduced SAM levels in DS compared to control mitochondria. The concomitant increased expression levels of SAMC in DS compared to control cells probably has to be ascribed to a mechanism aimed to compensate for the unmet demand of mitochondrial SAM. The significant decrease of SAM in mitochondria may suggest that in DS subjects a general depression of mitochondrial methylation capacity occurs (Fig. 4). The exact role of SAM into mitochondria is not well understood. The decrease of antioxidant defence in DS versus control mitochondria in the form of reduced GSH and total glutathione (GSSG + GSH) that we found, suggests that methylation in mitochondria may exert some kind of protective role or that, at least, reduced methyl availability in mitochondria exposes the organelle to more stressful conditions of oxidative stress. Its reduced availability in mitochondria could be related to the decreased level or activity of key energy enzymes, which has been documented in DS cells [15], because methylation is known to control gene and protein expression [22–25].

Methylation of proteins and nucleic acids in mitochondria is still a controversial issue since, thus far, the information reported on this
The topic is limited and little is known about its extent and role in mitochondrial function and disease. The notion that mtDNA could be methylated is supported by sparse evidence reported in mouse and man [26,27], but these findings have never been substantiated probably due to the poor detection method, which is mainly based on the bisulfide procedure. Evidence that tRNA and rRNA are

**Fig. 2.** LC-ESI-MS/MS (MRM mode with the monitor of 2 transitions) chromatogram of the nucleosides in a hydrolyzed mtDNA sample. The LC-ESI-MS/MS conditions are described in the Experimental Section. (A) Total ion chromatogram. (B) 5mdC was detected by monitoring m/z 242.1 - 126.1. (C) dC was detected by monitoring m/z 228.1 - 112.1.

**Fig. 3.** Control gels of mitochondrial DNA. (A) Purified mtDNA from lymphoblasts of DS subjects shows a band of about 15 kb. (B) Amplification of specific mitochondrial target sequence corresponding to the region from 14,018 to 14,508 of the mitochondria genome (primers ND6-A sense, 5'-TCCTCCTAGCATACCTGA and ND6-B antisense, 5'-GGATATCATAGCGATGC-3') is shown.

**Fig. 4.** Schematic overview of interaction between mitochondrial dysfunction, SAM carrier expression and some substrates of one carbon metabolism in DS. Downward and upward arrows indicate decreasing and increasing level, respectively. Lines are indicating the pathway. Abbreviations: SAM (S-adenosylmethionine), Met (methionine), Hcy (homocysteine), Cys (cysteine), GSH and GSSG (reduced and oxidized glutathione), THF (tetrahydrofolate), 5-CH3-THF (5-methyl-tetrahydrofolate), SAMC (S-adenosylmethionine carrier), IMM (inner mitochondrial membrane), and OMM (outer mitochondrial membrane).

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methylated in mammalian mitochondria has been recently reported [28,29]. The human mitochondrial transcription factor B1 (h-mtTFB1) has been shown to methylate a conserved stem-loop sequence in the human mitochondrial 12 S rRNA [30]. SAM is also required for basal transcription of mitochondrial DNA, probably via its interaction with the mitochondrial polymerase and mtTFB1 [31,32]. The presence of methylated bases in mtDNA that we found in human cells by applying a methodological approach, which overcomes the sensitivity problems of the bisulfite method, confirms the previous indications for methylation of mtDNA. The finding of mtDNA hypomethylation in DS cells, in which the mtDNA content is increased [15], could shed light on the mechanism of instability of DS mtDNA and help establishing a relationship between the genetically related abnormal methyl metabolism and the mtDNA mutations documented in DS subjects [33]. However, our findings are preliminary and first need to be confirmed by a larger set of samples. Our data supports the notion that aberration of the cytosolic methyl status results in a diminished methyl availability also in mitochondria that could in turn lead to mitochondrial dysfunction, which is an observation widely documented in DS. The elucidation of the physiological role of methylation in mitochondrial function will be the aim of future studies as it will provide important information concerning this genetic disorder.

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