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Manifold decrease of sialic acid synthase in fetal Down syndrome brain

Short Communication

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Summary. *Background:* Down syndrome (DS, trisomy 21) is the most common genetic cause of mental retardation. A large series of biochemical defects have been observed in fetal and adult DS brain that help in unraveling the molecular mechanisms underlying mental retardation.

Aims: As sialylation of glycoconjugates plays an important role in brain development, this study aimed to look at the sialic acid metabolism by measuring sialic acid synthase (SAS; N-acetylneuraminate synthase) in early second trimester fetal control and DS brain.

Results: In this regard, protein profiling was performed by two-dimensional gel electrophoresis coupled to matrix-assisted laser desorption/ionization mass-spectrometry followed by database search and subsequent quantification of spot using specific software. SAS, the enzyme catalyzing synthesis of N-acetyl-neuraminic acid (syn: sialic acid) was represented as a single spot and found to be significantly and manifold reduced ($P < 0.01$) in cortex of fetuses with DS (control vs. DS, 0.052 ± 0.025 vs. 0.012 ± 0.006).

Conclusion: The intriguing finding of the manifold decrease of SAS in DS fetal cerebral cortex as early as in the second trimester of pregnancy may help to explain the brain deficit observed in DS. Decreased SAS may well lead to altered sialic acid metabolism, required for brain development and, more specifically, for sialylation of key brain proteins, including neuronal cell adhesion molecule and myelin associated glycoprotein.

Keywords: Sialic acid synthase – Sialic acid – Two-dimensional gel electrophoresis – Down syndrome – Fetal brain

Introduction

Sialic acids represent a family of 3-deoxy-2-keto nine carbon sugars located in the non-reducing terminal ends of cell surface glycoclipids and glycoproteins of viruses, mammalian cells and some bacteria. These amino sugars play an indispensable role in myriad of biological activities, including development, regeneration and pathogenesis through a process termed as sialylation (Buttner et al., 2002; Hwang et al., 2002). Multiple sialic acid synthetic pathways have been identified in bacteria and eukaryotes (Hwang et al., 2002; Lawrence et al., 2000). Sialic acid aldolase found in both bacteria and mammals reversibly forms N-acetylneuraminic acid (NeuAc) from pyruvate and N-acetylmannosamine (ManNAc). Whilst in *E. coli* sialic acid synthase (SAS) directly forms NeuAc from phosphoenolpyruvate and ManNAc, a three-enzyme pathway has been identified in mammalian cells that converts ManNAc to NeuAc via the formation of ManNAc-6-P and NeuAc-9-P. This observation led to the notion that the human SAS (syn: N-acetyl neuraminate synthase, NeuAc-synthase, E.C. 4.1.3.19) unlike its *E. coli* homologue, uses phosphorylated sialic acids and thus probably represents the previously described sialic acid-9-phosphate synthase of mammalian cells (Lawrence et al., 2000). The sialic acids formed are then converted to the activated nucleotide sugar cytidine monophosphate NeuAC (CMP-NeuAc) by CMP-NeuAc synthase, which in turn serves as a substrate to a family of glycosyltransferases, the silayltransferases for sialylation of glycoconjugates.

Trisomy of human chromosome 21 is the major cause of mental retardation and other phenotypic abnormalities collectively known as Down syndrome (DS). The molecular mechanisms underlying the pathological alterations in DS are not known and it remains to be elucidated whether they are a reflection of developmental failure or degenerative processes. Although no characteristic

morphological alterations of DS brain have been noted in prenatal life (Unterberger et al., 2003), a series of biochemical changes have been reported in the literature that could explain why the brain of a child with DS develop differently from that of a normal one (for review see Engidawork et al., 2001, 2003; Lubec et al., 2002). To add to the body of evidence in the literature, a systematic protein hunting and profiling was performed on early second trimester DS fetal brain and here we report a manifold decrease of a protein identified as SAS.

Materials and methods

Brain tissue and sample preparation

Brain tissue (cerebral cortex) of aborted fetuses with DS (n = 8, male/female = 6/2, 19.81 ± 2.00 weeks of gestation) and controls (n = 7, male/female = 6/1, 18.79 ± 2.23 weeks of gestation) were kindly donated by Drs. M. Dierssen and J. Ferreres of Medical and Molecular Genetics Center-IRO, Hospital Duran i Reynals, Barcelona, Spain. Samples were taken in accordance with the guidelines of the local ethical committee. All the tissue samples were stored at −70° and the freezing chain was never interrupted.

Brain tissue was suspended in 0.5 ml of sample buffer consisting of 40 mM Tris, 5 M urea (Merck, Darmstadt, Germany), 2 M thiourea (Sigma, St. Louis, MO, USA), 4% CHAPS (3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate, Sigma), 10 mM 1,4-dithioerythritol (Merck), 1 mM EDTA (ethylenediaminetetraacetic acid, Merck), 1 mM PMSF (phenylmethanesulfonyl fluoride, Sigma) and 1 μg/ml of each pepstatin A, chymostatin, leupeptin and antipain. The suspension was sonicated for approximately 30 sec and centrifuged at 10,000 × *g* for 10 min and the supernatant was centrifuged further at 150,000 × *g* for 45 min to sediment undissolved material (Oh et al., 2005; Shin et al., 2005). The protein concentration of the supernatant was determined by the Coomassie blue method (Bradford, 1976).

Two-dimensional gel electrophoresis (2-DE)

2-DE was performed essentially as reported (Weitzdoerfer et al., 2002). Samples of 2 mg were applied on immobilized pH 3–10 nonlinear gradient strips (IPG, immobilized pH-gradient strips, Pharmacia Biotechnology, Uppsala, Sweden) in sample cups at their basic and acidic ends. Focusing started at 200 V and the voltage was gradually increased to 5,000 V at 3 V/min and kept constant for a further 24 h (approximately 180,000 kVh totally). The second-dimensional separation was performed on 9–16% SDS gradient polyacrylamide gels. The gels (180 × 200 × 1.5 mm) were run at 40 mA per gel. After protein fixation with 40% methanol containing 5% phosphoric acid for 12 h, the gels were stained with colloidal Coomassie blue (Novex, San Diego, CA, USA) for 48 h. Molecular masses were determined by running standard protein markers (Gibco, Basel, Switzerland), covering the range 10–200 kDa. Isoelectric point (pI) values were used as given by the supplier of the IPG strips. Excess of dye was washed out from the gels with water and the gels were scanned in an Agfa DUOSCAN densitometer (resolution 200). Electronic images of the gels were recorded using Adobe PhotoShop and Microsoft PowerPoint software.

Matrix-assisted laser desorption ionization mass spectroscopy (MALDI-MS)

MALDI-MS analysis was performed as described elsewhere with some modifications (Yang et al., 2004). Spots were excised with a spot picker and placed into a 96-well microtiter plate. Each spot was destained with 100 μl of 30% acetonitrile in 50 mM ammonium bicarbonate and dried in a speedvac evaporator. Each dried gel piece was rehydrated with 4 μl of 3 mM Tris-HCl, pH 9.0, containing 50 ng trypsin (Promega, Madison, WI, USA). After 16 h at room temperature, 7 μl of H_2O was added to each gel piece and the samples were shaken for 10 min. Four μl of 50% acetonitrile, containing 0.3% trifluoroacetic acid, the standard peptides des-Arg-bradykinin (Sigma, 904.4681 Da) and adrenocorticotropic hormone fragment 18–39 (Sigma, 2465.1989 Da), in water was added to each gel piece and shaken again for 10 min. Application of the samples was performed with a SymBiot I sample processor (PE Biosystems, Framingham, MA, USA). 1.5 μl of the peptide mixture was simultaneously applied with 1 μl of matrix, consisting of a saturated solution of α-cyano-4-hydroxycinnamic acid (Sigma) in 50% acetonitrile, containing 0.1% trifluoroacetic acid. The samples were then analyzed in a time-of-flight mass spectrometer (Reflex 3, Bruker Analytics, Bremen, Germany) using an accelerating voltage of 20 kV. Peptide matching and protein searches were performed automatically. The peptide masses were compared to the theoretical peptide masses of all available proteins from all species. Monoisotopic masses were used and a mass tolerance of 0.0025% was allowed. The algorithm used for determining the probability of false positive match with a given MS-spectrum is described elsewhere (Berndt et al., 1999).

Quantification of proteins and statistical analysis

Protein spots were outlined (first automatically and then manually) and quantified using the ImageMaster 2D Elite software (Amersham Pharmacia Biotechnology). Percentage of volume of a spot representing a particular protein was determined in comparison with the total proteins present in the area of interest. The software calculated percentage of spot volume in area of interest after subtraction of the background and these results are expressed as means ± standard deviation (SD). Inter-group differences were analyzed by the non-parametric Mann-Whitney U test. Correlations of age and post-mortem time and the spot density were examined with linear regression analysis. Statistical significance was considered at the $P < 0.05$. All statistical analyses were performed with the GraphPad Instat2 software, version 2.05.

Results

The spots were analysed by MALDI-MS following in-gel digestion (Fountoulakis, 2001). Peptide masses were matched with the theoretical peptide masses of all proteins of the SWISS-PROT database for identification. Internal standards were used to correct the measured peptide mass thus reducing the windows of mass tolerance and

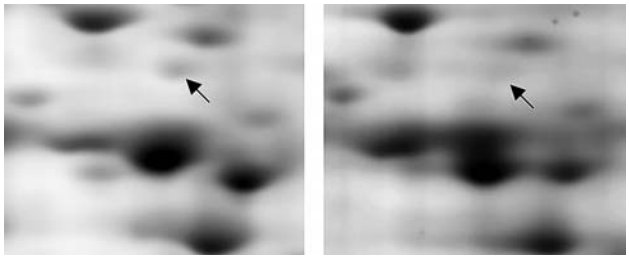

Fig. 1. Typical electrophoretic expressional pattern of sialic acid synthase (SWISS-PROT accession number Q9NR45) in controls and DS patients. Sialic acid synthase in cortex of fetuses with DS was downregulated about five-fold

increasing the confidence of identification. This procedure has led to identification of a single spot assigned to SAS (accession No. Q9NR45) with molecular size of 40 kDa, pI of 6.66, six matching peptides, score of 136 and probability of 2.6E-11. A representative control gel depicting the single spot for SAS is shown in Fig. 1. Quantification of this spot revealed a significant decrease ($P = 0.0043$) in mean levels of SAS in DS brain (0.012 ± 0.006) compared to controls (0.052 ± 0.025). Regression analysis failed to show any correlation between age, sex and postmortem interval and levels of SAS in both control and DS groups (data not shown).

Discussion

About five-fold reduction of SAS levels in fetal DS brain is the major finding of the present work and this may have serious consequences on several pathways of sialic acid metabolism as well as on sialoproteins known to play pivotal roles in brain development and function, including nerve branching, fasciculation, synaptic plasticity, axonal outgrowth and stability of the neuronal matrix (Minana et al., 1998; Matsuhashi et al., 2003). SAS is a 40 kDa enzyme encoded on chromosome 9, ruling out the decrement to be a direct consequence of trisomy 21.

Carbohydrate-receptor interactions participate in numerous cell–cell recognition events in eukaryotes (Brockhausen, 1999; Blackhall et al., 2001). Of the nine monosaccharides that constitute mammalian polysaccharides, the common terminal residue sialic acid stands out as a major determinant of cell–cell interactions and is the major binding determinant of Siglecs, a family of sialic acid-binding lectins, involved in many cellular processes, such as signaling (Crocker and Varki, 2001). Myelin-associated glycoprotein (MAG, Siglec-4) is a transmembrane cell adhesion molecule expressed by myelinating glial cells and it stabilizes myelin-axon interactions, probably through inhibition of outgrowth of neurons, by binding to complimentary ligands on the axolemma. The sialic acid binding site on MAG creates the potential to bind to many molecules bearing the correctly presented terminal sialic acid so that MAG could exert its action. The likely candidates are gangliosides GD1a and GT1b, which are the major sialoglycoconjugates on mammalian axons (Vinson et al., 2001; Sun et al., 2004). Studies on knockout mice support the theory that interaction between MAG and gangliosides mediates the effects of MAG on neurons. The phenotype of MAG-deficient mice closely resembles that of mice lacking complex gangliosides and includes decreased central myelination and axonal degeneration (Sheikh et al., 1999). More importantly, these effects increase in intensity as the mice age (Sun et al., 2004). This is consistent with age-related changes in levels of gangliosides (Brooksbank and McGovern, 1989; Brooksbank et al., 1989) as well as dysmyelination and axonal degeneration seen in adult DS brain (Vlkolinsky et al., 2001). Thus, it is conceivable that lack of sialic acid secondary to decreased SAS may create a signal gap between MAG and ganglosides, leading to delayed myelination in DS. It was observed that MAG phosphorylation was significantly downregulated in the presence of S100ß (Kursula et al., 2000), which is located on chromosome 21 and is highly overexpressed in DS (Jorgensen et al., 1990). Thus, posttranslational modification of MAG is another important aspect that might contribute to deranged myelination apart from disturbance of the sialic acid metabolism.

Neural cell adhesion molecule (NCAM) is a major cell adhesion molecule in the central nervous system and is thought to operate by promoting cell–cell interactions by homophilic binding of NCAM molecules on apposing membranes. During central nervous system development, the majority of NCAM is highly polysialylated and has a major role in axon pathfinding, target innervation, and fasciculation. Polysialylation of NCAM has been reported to significantly reduce the ability of NCAM molecules to interact and as a result enhances cell migration (Doherty and Walsh, 1996; Hu et al., 1996). Evidence supporting this notion comes from the observation that replacing sialic acid with unnatural sialic acids significantly reduced the ability of neurons to project neurites in NCAM expressing HeLa substartum cells (Charter et al., 2002). Studies in brains of newborns and older infants with DS have revealed relatively delayed myelination, fewer neurons, lower neuronal density and distribution, and abnormal synaptic density and length, caused probably by prenatal abnormal neuronal migration and retarded synaptogenesis (Wisniewski and Schmidt-Sidor, 1989; Wisniweksi, 1990). Decreased expression of SAS in fetal DS brain is thus in line with the view of abnormal neuronal migration and may provide a possible molecular mechanism for its occurrence.

In conclusion, the finding of an inefficient sialic acid metabolism in fetal DS brain may provide explanation, at least in part, for the chain of events leading to progressive postnatal neuronal development defects that eventual culminate in mental retardation. The report that Lewis-X fraction in patients with DS has a higher sialic acid reactivity and could be responsible for mental retardation (Masuda et al., 1997) signifies the importance of sialic acid metabolism in the genesis of mental aberration.

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