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Journal of Neuroscience Methods 161 (2007) 223–233

JOURNAL OF NEUROSCIENCE METHODS

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A spontaneously immortalized Schwann cell line to study the molecular aspects of metachromatic leukodystrophy

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Received 13 October 2006; received in revised form 16 November 2006; accepted 19 November 2006

Abstract

The arylsulfatase A (ASA)-deficient mouse is a murine model of human metachromatic leukodystrophy (MLD) caused by a genetic defect in the ASA gene. Deficiency of ASA causes accumulation of cerebroside-3-sulfate (sulfatide) in visceral organs and in the central and peripheral nervous system, which subsequently causes demyelination in these areas. To investigate further the cellular pathomechanism of MLD, we established spontaneously immortalized Schwann cell lines from ASA-deficient mice. Cells showed marked sulfatide storage in the late endosomal/lysosomal compartment. This sulfatide accumulation can be further increased by external treatment with sulfatide using a lipid based transfection reagent as a cargo. The accumulated sulfatide was degraded in response to ASA treatment and first examination revealed that alteration on the molecular level found in ASA-deficient mice can also be observed in the presented cell culture model. Hence, these cells could be a suitable model to study MLD at a molecular level.

Keywords: Schwann cells; Metachromatic leukodystrophy; Sulfatide; Arylsulfatase A

1. Introduction

Sulfatide is a major component of myelin. It is degraded by the lysosomal enzyme arylsulfatase A (ASA). The genetic deficiency of this enzyme results in defective desulfation of sulfatide and other 3-*O*-sulfogalactosyl containing glycolipids which causes accumulation of this molecules in the lysosomal compartment. The corresponding human disease is called metachromatic leukodystrophy (MLD), which is characterised by progressive demyelination accompanied by severe neurological symptoms. In patients, macroscopically, reduced volumes of white matter and in severe cases spongiform or cystic degeneration are noted. Microscopically loss of myelin sheaths, a reduction in number of oligodendrocytes and accumulation of metachromatic granules are observed (for reviews see von Figura et al., 2001). In the peripheral nervous system bloated Schwann cells containing tuff stone like inclusions can be detected. Reductions in large as well as small myelinated fibre populations were reported. These observations are accompanied by severe reduction in conduction velocities of peripheral nerves in MLD patients (Bindu et al., 2005; Cameron et al., 2004). Therefore, this lysosomal storage disease beside its impact on the central nervous system exhibits also a clear peripheral nervous system component which can be useful in the diagnosis of MLD (Olsson and Sourander, 1969; Vos et al., 1982).

The early pathological changes of MLD and course of development of pathology are unknown. A mouse model for MLD has been generated through specific disruption of the murine ASA gene. The phenotype of ASA-deficient mice is mild, but recapitulates early stages of the disease progression in MLD (Gieselmann et al., 1998; Hess et al., 1996). To date, it is not clear how sulfatide storage affects metabolism of a cell at the molecular level. The investigation of pathogenetic mechanisms would be largely facilitated by investigation of sulfatide storing cell culture models. The only system of sulfatide storing cells available today uses primary renal distal tubule cells (Klein et al., 2005). To a certain degree it has proven to be useful studying some molecular aspects of ASA-deficiency (Klein et al., 2005; Saravanan et al., 2004). However, in vivo MLD patients as well as ASA-deficient mice do not show any severe renal phenotype.

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doi:10.1016/j.jneumeth.2006.11.009

Therefore, a cell culture model using the most affected cell types in MLD like oligodendrocytes or Schwann cells is highly important for understanding the molecular mechanisms of the pathophysiology of this lipid storage disorder.

Here, we report the establishment of a cell culture model of sulfatide storing Schwann cells. We describe a simple method for the Schwann cell preparation from sciatic nerves of 2-week-old ASA-deficient mice using a combination of previously published methods. The Schwann cells isolated were spontaneously immortalized. The resulting Schwann cell line is transfectable, stores sulfatide in the late endosomal/lysosomal compartment and responds to external ASA enzyme treatment. Therefore, this cell line should be helpful in understanding the influence of sulfatide storage on cellular metabolism and can serve as a model system to study MLD at the molecular level.

2. Materials and methods

2.1. Materials

Unless otherwise specified, chemicals were procured from Serva (Heidelberg, Germany), Sigma (Deisenhofen, Germany), GIBCO (Germany), Roche (Basel, Switzerland) or Merck (Darmstadt, Germany).

TRIzol™ reagent was from Invitrogen (Karlsruhe, Germany), bovine pituitary extract (BPE) from Upstate Biotechnology (NY, USA), RevertAid H Minus first strand cDNA synthesis kit™ from Fermentas (Leon-Rot, Germany) and Transfectin® from Bio-Rad (Munich, Germany). Antibodies against LAMP-1 (1D4B) and LAMP-2 (GL2A7) were procured from the developmental studies hybridoma bank (Iowa City, IA, USA). Antibodies against p75 and sulfatide (Sulf-1) were provided by S. Kelm (CBIB, University Bremen) and P. Fredman (Molndal, Sweden), respectively. All peroxidase and fluorescently labelled secondary antibodies were from Jackson IR Laboratories (West Grove, PA, USA). Recombinant human arylsulfatase A was produced like described elsewhere (Matzner et al., 2005).

2.2. Cell culture

Animals which were used in this study to prepare Schwann cells were derived from the same breed as previously described (Hess et al., 1996). Some modifications from the method of Brockes et al. (1979) were used to prepare the Schwann cells from sciatic nerves. Primary cells from four pairs of sciatic nerves of 2-week-old ASA-deficient mice were prepared by chopping the excised sciatic nerves into 1 mm pieces in a small petri dish with Hank's balanced salt solution (Komiyama et al., 2003). Chopped material was collected as a pellet by centrifugation at $800 \times g$ for 3 min. The pellet was incubated for 50 min with 2.5% trypsin without EDTA and 0.6% collagenase type I at 37 °C in a humidified 5% CO_2 incubator with intermittent shaking. The dissociated cells and the undissociated tissues were passed 4–5 times through a Pasteur pipette and 8–10 times through a 23 G needle. Protease activity was neutralized using advanced Dulbecco's modified essential medium (ADMEM) containing 10% FCS, 50 units/ml penicillin, 50 μg/ml streptomycin and 2 mM L-glutamine. Cells were collected by centrifugation at $1800 \times g$ for 10 min without brake. The cell pellet was resuspended in proliferation medium (ADMEM containing 10% FCS, 100 μg/ml BPE, 2 μM forskolin, antibiotics and L-glutamine as above) (Weiner and Chun, 1999), seeded on 0.1% gelatine coated 6-well plates and incubated at 37 °C in a humidified 5% CO_2 incubator for 5–6 days without any disturbance. The supernatant with unattached cells was collected, centrifuged at $1800 \times g$ for 5 min. The cell pellet was resuspended in the proliferation medium seeded similarly as above in two more wells and incubated under the same conditions for further 3–4 days (Komiyama et al., 2003). In this so-called second seed, almost 80% of cells morphologically resembled a Schwann cell like phenotype. Similarly third and fourth seedings were carried out which led up to 90% Schwann cell purity in these wells. Up to 80% of contaminating cells were killed by holding the cells for 2 weeks in ADMEM without FCS and replenishing with fresh medium once in 4–5 days (Komiyama et al., 2003). The cultures were passaged using accutase (Sigma) for gentle dissociation and propagation once in 5 weeks and maintained in culture for about 6 months until cells spontaneously immortalised. Finally, the cells were purified by isolating pure clones using a cloning plate. Four isolated clones were propagated in proliferation medium and aliquots were frozen in liquid nitrogen for further examination. All clones isolated showed a similar spindle like morphology. The clone chosen for further examination has a doubling time of about 48 h in full proliferation medium. Withdrawal of either FCS or bovine pitiuary extract resulted in a more or less complete growth arrest of the cells.

2.3. Alcian Blue staining

The cells were fixed in 4% paraformaldehyde (PFA) and stained with Alcian Blue similar to the method described for tissue sections (Matzner et al., 2002; Schott et al., 2001; Scott and Dorling, 1965). Cells were incubated with 0.05% (w/v) Alcian Blue in 0.025 M sodium acetate buffer, pH 5.7, containing 0.3 M $MgCl_2$ and 1% (w/v) PFA for 2–3 weeks. The cells were rinsed four to five times with physiological buffer to remove traces of unbound stain and examined microscopically.

2.4. Immunohistochemistry

Cells intended for immunohistochemistry were grown on 0.1% gelatin coated cover slips in a 24-well plate. For immunofluoresence against sulfatide, lysosome associated membrane protein (LAMP) and p75, cells were fixed in 4% PFA at room temperature for 30 min and incubated with 0.5% sodiumborohydrate for 10 min to quench cellular autofluorescence. In case of S100, the cells were postfixed in methanol for 10 min at −20 °C after PFA treatment. After washing three times with PBS, cells were treated with 0.3% Triton X-100 (TX-100) for 5 min, washed and blocked with 2% normal goat serum for 60 min. Primary and secondary antibody dilutions were made in 0.5% goat serum and incubated for 1–2 h at room temperature. For p75 staining, goat serum was replaced with 2% donkey