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Chapter 5

A NOVEL ASSAY FOR THE PRENATAL DIAGNOSIS OF SJÖGREN–LARSSON SYNDROME

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Chapter 5

A novel assay for the prenatal diagnosis of Sjögren-Larsson syndrome

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Summary
Sjögren-Larsson Syndrome (SLS) is a metabolic disorder characterized by ichthyosis, mental retardation and spastic diplegia or tetraplegia. The biochemical defect has been identified as a deficiency of Fatty Aldehyde Dehydrogenase (FALDH), which is part of an enzyme complex that converts fatty alcohols into fatty acids. Making use of the finding that FALDH is also involved in the degradation of phytol, we set up an enzymatic assay for the prenatal diagnosis of SLS in cultured chorionic villus fibroblasts (CVF) based on a deficiency in the conversion of phytol to phytanic acid. FALDH activity was assessed by incubating fibroblast homogenates with phytol in the presence of NAD\(^+\), followed by hexane extraction of the samples and quantitation of phytanic acid production by gas chromatography-mass spectrometry (GC-MS). FALDH activity could be detected in cultured CVF cells derived from control fetuses and the activity was found to be 90% deficient in cultured CVF cells derived from an affected SLS fetus. The new assay described in this paper has advantages over previous assays and allows for the easy and reliable diagnosis of SLS.

Introduction
Sjögren-Larsson Syndrome (SLS; MIM 270200) is an autosomal recessive disorder of fatty alcohol metabolism, characterized by ichthyosis, spastic di- or tetraplegia and mental retardation. In most patients ichthyosis is present at birth, while the latter two symptoms develop somewhat later in the first few years of infancy (1). Fatty alcohol metabolism is impaired due to a defect in the enzyme Fatty Aldehyde Dehydrogenase (FALDH, EC 1.2.1.48), which is a component of the fatty alcohol:NAD\(^+\)-oxidoreductase (2, 3). This enzyme complex catalyzes the conversion of fatty alcohols into fatty acids and has a preference for long-chain fatty alcohols and presumably plays

Figure 1. Conversion of phytol to phytanic acid. The reaction takes place in two NAD\(^+\)-dependent steps, the first catalyzed by an unknown alcohol dehydrogenase (ADH) and the second by FALDH.
Diagnostic assay of SLS in CVF

a role in the cycle between fatty alcohols and fatty acids (4). The consequence of FALDH deficiency therefore, is an accumulation of fatty alcohols in tissues and plasma of SLS patients (5). In addition to this, FALDH was also found to be involved in the degradation of leukotrienes, leading to increased levels of leukotriene B, in SLS patients (6).

Recent findings in our laboratory have uncovered yet an additional function of FALDH, in the breakdown of phytol (7). The branched-chain fatty alcohol phytol (3,7,11,15-tetramethylhexadec-2-en-1-ol) is part of the chlorophyll molecule and, once released from it, can be metabolized into phytanic acid, a fatty acid involved in the pathogenesis of Refsum disease (8). The breakdown of phytol takes place in a few steps (shown in Fig. 1) and the production of one of the intermediates, phytanic acid, was shown to be deficient in fibroblasts derived from SLS patients upon incubation with phytol (7). From these studies it became apparent that phytol was specifically metabolized by FALDH and might therefore be used as a diagnostic assay for FALDH deficiency (9).

Since the enzymatic methods that have been described so far for prenatal diagnosis of SLS make use of radio labeled substrates or suffered from high background activity, we set out to develop a GC-MS method based on phytol degradation. Here we describe an assay in which cultured chorionic villus biopsy fibroblasts (CVF) are incubated with phytol, followed by measurement of phytanic acid that is produced to determine FALDH activity. In cultured CVFs derived from a fetus affected by SLS, residual activity was found to be minimal, which makes it a suitable assay for the prenatal diagnosis of this syndrome.

Patients and Methods

Chorionic villus biopsy material was collected from a woman who had previously given birth to a patient with SLS. Detailed studies in fibroblasts from the index patient had shown deficient FALDH activity and molecular analysis had shown distinct mutations in the ALDH10 gene as was described previously (patient 1 in: 10).

Cell culture

CVF cells were cultured in Nutrient mixture Ham's F-10 with L-glutamine and 25 mM HEPES (Gibco, Invitrogen) supplemented with 20% fetal calf serum (Gibco), penicillin (100 U/ml) and streptomycin (100 μg/ml, Gibco) at 37°C and with 5% CO₂. Cells were harvested using trypsin and stored as cell pellets at -80°C.

Biochemical assay

Phytol degradation was performed as described (chapter 4 or reference 9). Briefly, cells were taken up in phosphate buffered saline and homogenized by sonication on ice. The incubation mixture consisted of 100 μg/mL protein, 50 mM glycine buffer (pH 9.2), 1 mM NAD⁺, 0.1% Sodium Cholate and 1 mg/ml methyl-β-cyclodextrin (Fluka) in a total volume of 500 μL. Reactions were performed at 37°C and initiated by the addition of 200 μM phytol dissolved in dimethyl sulfoxide (DMSO). After 60 minutes, the incubation was terminated by the addition of 100 μL 1 M HCl. Then 2 mL of hexane was added, after which the organic layer was extracted and evaporated to dryness under nitrogen at 40°C. The sample was then derivatized with N-tert-butylidimethylsilyl-N-methyl-trifluoroacetamide (MTBSTFA) and analyzed on an Agilent Technologies model 5890/5973 GC-MS system equipped with a CPsil 19CB capillary column (25 m X 0.25 mm I.D., film thickness 0.25 mm, Varian), with electron impact ionization applied at 70 eV. MS acquisi-
situation was performed in the single ion monitoring mode, monitoring the [M-57]⁺ ions of the various compounds.

**Results**

Earlier we reported on the development of a specific assay for the diagnosis of SLS based on phytol degradation (7, 9). To evaluate whether this could also be used for activity measurements in cultured CVF cells, we incubated CVF cell homogenates derived from control pregnancies with phytol in the presence of NAD⁺. At the chosen incubation conditions, activity was linear in time up to 2 hours (Fig 2A). The activity was linear with protein up to at least 0.2 mg in the assay (Fig 2B).

Since these results indicated that conversion of phytol into phytanic could indeed be measured in cultured CVF cells, we performed the analysis in cells derived from a fetus at risk for SLS. The results as depicted in Fig. 2C show that activity was deficient compared to 6 different control samples.

![Graphs showing characteristics of phytol degradation in cultured CVF cells.](image)

**Figure 2.** Characteristics of phytol degradation in cultured CVF cells. Graphs show the dependence of the reaction to (A) incubation time and (B) amount of protein. (C), Phytol degradation activity in cultured CVF cell homogenates derived from controls and a SLS-affected fetus. The control values represent the mean ± SD of the activity expressed in nmol/min/mg protein (n=6).

**Discussion**

In our experience, the different biochemical assays for the prenatal diagnosis of SLS described in literature so far (11) proved either to be cumbersome due to the use of radio labeled substrates, or to yield ambiguous results. The latter problem frequently occurs in assays that measure the production of NADH by fluorescence as a measure of enzyme activity rather than measuring the reaction product directly, which makes them susceptible to background fluorescence caused by non-specific reactions occurring in the cell homogenate. In addition to this, because of substrate overlap, other aldehyde dehydrogenases present in cell homogenates can contribute to background activity (12). Together with the fact that in cultured CVF cell homogenates the activity of fatty alcohol:NAD⁺-oxidoreductase as well as FALDH itself is even lower than in fibroblasts (11), results can often be ambiguous.

For this reason, we extended the assay based on the degradation of phytol we set up for fibroblast homogenates (9) to cultured CVF cells. The advantage of the new assay is that phytol is a relatively specific substrate for FALDH reducing most background
activity. Furthermore, since this assay makes use of GC-MS analysis of metabolites, direct quantification of the reaction product, phytanic acid, can be done. The activity of phytol degradation was high enough to be measured reliably in cultured CVF cells and residual activity found in the material derived from the fetus affected with SLS was around 13%. Therefore, we conclude that this assay is suitable for reliable prenatal diagnosis of SLS.

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References
