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Pathomechanisms Underlying X-Adrenoleukodystrophy: A Three-Hit Hypothesis

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Abstract

X-adrenoleukodystrophy (X-ALD) is a complex disease where inactivation of ABCD1 gene results in clinically diverse phenotypes, the fatal disorder of cerebral ALD (cALD) or a milder disorder of adrenomyeloneuropathy (AMN). Loss of ABCD1 function results in defective beta oxidation of very long chain fatty acids (VLCFA) resulting in excessive accumulation of VLCFA, the biochemical “hall mark” of X-ALD. At present, the ABCD1-mediated mechanisms that determine the different phenotype of X-ALD are not well understood. The studies reviewed here suggest for a “three-hit hypothesis” for neuropathology of cALD. An improved understanding of the molecular mechanisms associated with these three phases of cALD disease should facilitate the development of effective pharmacological therapeutics for X-ALD.

Keywords

axonal degeneration; demyelination; neuroinflammation; oxidative stress; peroxisomes; plasmalogens; VLCFA; X-adrenoleukodystrophy

Introduction

With an incidence of 1 in 17 000 males, X-linked adrenoleukodystrophy (X-ALD, OMIM number 300100) is the most common monogenic leukodystrophy and peroxisomal disorder. It is characterized by axonopathy in the spinal cord, demyelination in the cerebral hemispheres and adrenal insufficiency. X-ALD patients accumulate saturated, and to a lesser extent, monounsaturated, very long chain fatty acids (VLCFA) in plasma and tissues, most notably in the brain and adrenal cortex (14,40,59).

All X-ALD patients present with mutations in the ABCD1 gene, which encodes a peroxisomal adenosin triphosphate (ATP)-binding cassette transporter protein (ABCD1, ALDP) (41). ABCD1 has been proposed to play a crucial role in transport of VLCFA, or their Coenzyme A (CoA) derivatives, into peroxisomes. This is based on the VLCFA accumulation and their defective beta oxidation of VLCFA and on the role of the ABCD

transporter orthologs in the peroxisomal import of fatty acids in yeast (26,54). Recent studies support this hypothesis as the overexpression of human ABCD1 in *Saccharomyces cerevisiae* allows the import of several acyl-CoA esters of long chain and VLCFA, such as C18:1 ω 9, C16:0, C22:0, and C24:6 ω 3, into peroxisomes (60).

X-ALD is a complex inherited disease where the same mutation in the ALD gene (ABCD1) can lead to clinically very distinct phenotypes, a fatal childhood cerebral ALD (cALD) or the relatively benign adult disease of adrenomyeloneuropathy (AMN) (40,59).

cALD is characterized by progressive cerebral demyelination with a strong inflammatory response in the white matter leading to neurodegeneration and death often before the patient reaches adolescence.

AMN affects adults (second to fourth decade) and is characterized by a pure myelopathy and peripheral neuropathy. The neuropathological hallmark of AMN is an axonopathy (resembling spastic paraparesis or spastic paraplegia) without significant myelin degeneration or neuroinflammation. However, about 35% of AMN patients subsequently develop cerebral demyelination: these patients share the same poor prognosis as children with cerebral ALD.

Frequently, the different X-ALD phenotypes occur within the same family as a result of an identical ABCD1 mutation. No phenotype–genotype correlations have been established so far, which suggests that of modifier genes or environmental/epigenetic/stochastic factors modulate the clinical outcome of the disease. The molecular events that trigger the transition from the metabolic phase, which is the accumulation of VLCFA that is already found *in utero*, to neuroinflammation and demyelination in the brain in cALD or to axonal degeneration in spinal cords in AMN are largely unknown. Moreover, many AMN patients affected by axonal degeneration later develop inflammatory demyelination leading to death for reasons that remain obscure.

Until recently, the generally held view on the pathogenesis of X-ALD was that the loss of function of ALD protein (ALDP) causes the accumulation of VLCFA, which would be incorporated in an anomalous manner into complex lipids such as gangliosides or phosphatidylcholine (20,57). These structural alterations in the myelin lipidic components would cause destabilization of myelin sheath and onset of demyelinating pathology. Axonal degeneration could respond to the same cause because integrity of the cellular membrane is a prerequisite for myelin–axonal interaction.

However, the X-ALD mouse model, a classical knockout of the ABCD1 gene, do not exhibit signs of demyelination in spite of accumulation of VLCFA in the brain and the nervous tissue (16,37). Instead, ABCD1 null mice presents with a late onset neurodegenerative phenotype with defective motor coordination, locomotor activities and slower nerve conduction velocity at around 20 months of age. First immunohistochemical evidence of axonal degeneration and myelin pathology was detectable in the spinal cord after 16 months of age but not in the brain. Later in life, it was accompanied by microgliosis and astrogliosis (11,52,53).

The phenotype of ABCD1-deficient mice mimics features of human AMN, thus providing a model for investigating the pathogenesis of this disease and for evaluation of efficacy of pharmacological or gene therapeutic approaches (53). The lack of disease progression from the metabolic abnormalities and axonal degeneration to cerebral demyelination and neuroinflammation suggest that in addition to loss of ALDP function and VLCFA excess, it is likely that other factors play a critical role for X-ALD disease expression that differ in a mouse and a man.

The Cytotoxicity of VLCFA

The exact mechanism that links the VLCFA excess to axonal degeneration in AMN or to inflammation and demyelination in cALD remains elusive.

VLCFA are usually found as constituents of complex lipids, such as ganglioside, phosphatidilcholine and cholesterol ester fractions of brain myelin, and are also found in the proteolipid protein. In particular, gangliosides and phosphatidilcholine have been proposed as trigger of the immune response in cerebral ALD. Theda and colleagues had observed greatest VLCFA excess (16-fold higher) accumulated in the phosphatidilcholine fraction in intact regions of brain white matter (57). Recently, highest concentration of VLCFA were also observed in lysophosphatidilcholine (lysoPC) in cALD brain (28). The gangliosides of an X-ALD brain contain 28% to 50% of fatty acids with chains exceeding 22 carbons (20). Such VLCFA are absent from gangliosides of control individuals. Of note, immunologic properties of gangliosides vary with their fatty acid composition, and they have been implicated in a variety of immunological brain diseases (44).

Studies of VLCFA cytotoxicity using free fatty acids might bring in a limitation for interpretation of data as the largest pool of fatty acids in the cells are not found free but complexed to phospholipids and other lipidic species. Secondly, in addition to eliciting an immune response, this incorporation of VLCFA to complex lipids might destabilize cell membranes. It is well established that hexacosanoic acid (C26:0) alters the physiological properties of membranes. Microcalorimetric studies revealed that inclusion of C26:0 in a model membrane would disrupt its structure (27). Moreover, turnover of C26:0 from phospholipid membranes is 10 000 times slower than that of long and medium-chain fatty acids (27). Indeed, the microviscosity of erythrocyte membranes was reported to be increased in X-ALD patients (35), an abnormality that was replicated in adrenocortical cell membranes exhibiting increased microviscosity and a decreased Adrenocorticotrophic hormone (ACTH)-stimulated cortisol secretion as a proof of impaired membrane function (63). Therefore, it is tempting to speculate that similar alterations might be found in the cellular membranes of neural cells and even of membrane of organelles, thus impairing essential functions that would contribute to the pathogenesis of AMN and cALD (reviewed in Powers *et al* (47)).

Toxicity mechanisms of VLCFA excess were also investigated using various rat primary neural cell cultures reporting changes in the cellular calcium levels, alterations in mitochondrial functions and cell death (25). At 20 mmol/L and higher, VLCFA (C22:0, C24:0 and C26:0) induce cell death of oligodendrocytes and astrocytes. VLCFA induced increase in intracellular calcium concentrations in oligodendrocytes, being this cell type more sensitive than astrocytes and neurons. In a similar setting, the mitochondrial membrane potential was markedly decreased, in particular by C22:0. Mitochondria respiration, in absence of ADP (state 4) or presence of ADP (active respiration, state 3) was also inhibited. Consequently, the authors concluded that VLCFA caused cell death by disturbing mitochondria metabolism and calcium homeostasis. Production of reactive oxygen species (ROS) was not detected in this system, which used normal cells instead of ABCD1 deficient cells.

In contrast, a parallel study using human fibroblasts demonstrated that hexacosanoic acid excess (doses up to 100 μ M) generates ROS together with depletion of reduced glutathione and a decrease in mitochondrial membrane potential in absence of cell death (17). Disturbance in mitochondria membrane potential seemed common to both types of cultured cells, neural and fibroblasts. Production of free radicals might lead to oxidative stress when antioxidant defenses are overwhelmed. As an outcome, oxidation damage of

macromolecules such as DNA, RNA or proteins lead to detrimental consequences for cell and organisms. Indeed, incubation of fibroblasts with C26:0 excess generated oxidative damage to proteins, arising from metal-catalyzed oxidation and glycooxidation/lipoxidation, as detected with mass spectrometry techniques. This oxidative damage to proteins occurs in X-ALD fibroblasts but not in control fibroblasts upon C26:0 excess. Moreover, the loss of ABCD1 gene function also sensitizes fibroblasts to death upon glutathione depletion, in their culture medium, thus indicating that ABCD1 dysfunction alters oxidative stress homeostasis (17). The reversal of oxidative lesions caused by VLCFA excess reversed *in vitro* by vitamin E offers therapeutic hope as discussed in this symposium (4).

A recent, study has addressed the cytotoxicity of VLCFA complexed to phospholipids in particular lysophosphatidilcholine (lysoPC C24:0) (12). In this study, Eichler and coworkers injected lysoPC C24:0 into brain parietal cortex of mice, causing microglial activation and apoptosis. This toxicity did not occur when lysoPC C16:0 was used, indicating a fatty chain length dependent cytotoxicity. Of note, the C26:0 content of lysoPC fraction in peripheral blood is being currently validated as biomarker for the newborn screening of X-ALD (29).

The First Hit: Metabolic Disease of X-ALD and Axonal Degeneration

Underscoring the described *in vitro* results, evidence of oxidative damage was also documented in human X-ALD brain: oxidative lesions were found in the form of the lipid peroxidation product HNE (hydroxy-nonenal) (50). Both AMN and cALD patient had increased lipid peroxidation (TBA-RS) and decreased tissue nonenzymatic antioxidants (TAR) (7,61). Recently, damage to proteins has also been reported in peripheral mononuclear cells from X-ALD patients (19).

Because oxidative stress is involved in a wide variety of neurological disorders, it may be considered a common event in neurodegenerative pathway that occurs in late stages of the disease and is simply epiphenomenal or a consequence of tissue damage. Therefore, the use of antioxidants is being questioned as a valid therapeutic strategy for treating multifactorial diseases in which multiple and parallel pathways contribute to neurodegeneration, in Alzheimer disease, Parkinson disease or multiple sclerosis. In X-ALD, however, we have recently reported evidence suggesting that oxidative stress is an early etiologic factor contributing to disease pathogenesis in X-ALD mice do not exhibit an overt locomotor phenotype until 20 months of age in spite of early signs of axonal pathology of APP. Synaptophysin deposition in axonal swellings of spinal cord can be detected at 15 months of age along with ultrastructural signs of myelin and axonal damage in peripheral nerves (52).

Recently, we have also observed oxidative damage to proteins and lipids of the same nature, observed in lesions produced by excess of VLCFA in X-ALD fibroblasts, as an early biochemical feature of the neurodegenerative cascade in the spinal cord of ABCD1 null mice at 12 months, prior to disease onset. Interestingly, as early as 3.5 months of age, the malonaldehyde-lysine (MDAL) marker, a consequence of lipoxidative damage to proteins, is detected as the most predominant type of lesion to proteins derived from lipid oxidation adducts (17). Moreover, the degree of VLCFA accumulation parallel the levels of lipoxidative damage to proteins as recently seen in a double knockout mouse deficient for both ABCD1 and ABCD2 genes (19,53). It correlates with an earlier disease onset of more severe neurodegenerative phenotype of the double mutant mice, with axonal damage, locomotor and behavioral abnormalities and peripheral nerve conduction impairment (13,53). ABCD1 and ABCD2 share overlapping functions in the beta oxidation of hexacosanoic acid (13,19) and only double mutants present accumulation of C26:0 in serum (18). However, ABCD2 seems to have higher affinity to other fatty acid species, such as C20:0, C22:1 ω 9 and the precursor of DHA (C24:6 ω 3) (18,36). Of note, the double mutant

ABCD1/ABCD2 presents occasionally and at very old ages with inflammatory infiltrates of T-lymphocytes, a feature absent in ABCD1 or ABCD2 single mutants (53).

In addition to VLCFA abnormality, the functional loss of ABCD1 in X-ALD results in an additional defective peroxisomal function, the synthesis of plasmalogens (vinyl ether lipids with antioxidant activity). Accordingly, decreased plasmalogens were reported in X-ALD patient brains along with increased levels of VLCFA (33,56). Plasmalogen biosynthesis utilizes acyl-CoA generated by the peroxisomal beta oxidation pathway (23,24). Presumably, ABCD1 inactivation results in lower amounts of plasmalogens because of lack of substrates (acyl-CoA). Therefore, metabolic derangements in levels of VLCFA and plasmalogens might act synergistically in increasing oxidative stress in X-ALD.

As oxidative stress was reported to be directly responsible for axonal degeneration in cultured primary neurons (15,51) or *in vivo* in an experimental autoimmune encephalomyelitis (EAE) model (2), it seems reasonable to propose oxidative burden (direct consequence of peroxisomal metabolic impairment) as a first hit in the physiopathogenesis of axonal damage in X-ALD (Figure 1).

The Second Hit: Induction of Inflammatory Phenotype of cALD

The transition from the metabolic disturbance linked to oxidative stress to neuroinflammatory disease of cALD is driven by the “second hit.” The molecular events associated with the transition from a relatively benign metabolic disease to a fatal neuroinflammatory disease in cALD are not well understood at present.

A recent set of data provides some insights into the possible role of ABCD1 dysfunction in triggering of inflammatory response in glial (astrocytes and C6 glial cells) cells. Studies using rat primary astrocytes silenced for ABCD1 and/or ABCD2 reported accumulation of VLCFA, reduced plasmalogens, increased reactive oxygen species production, expression of enzymes of eicosanoid pathway (5-lipoxygenase, cyclooxygenase-2) and induction of inflammatory mediators such as cytokines (TNF-alpha, IL-1beta) and inducible nitric oxide synthase (iNOS) as well as alterations of peroxisomal metabolism (56). The validity of the relationship between VLCFA accumulation leading to inflammatory response was further established by down regulation of these changes by reducing the VLCFA load by treatment with Lorenzo's oil (mixture of oleic and erucic acids). Lorenzo's oil reduces the levels of VLCFA by competing for enzymes for fatty acid chain elongation. Another study reported that silencing of ABCD3 gene in C6 glial cell line also induces expression of inflammatory mediators (8). Astrocytes silenced for ABCD1 and/or ABCD2 and C6 glial cells silenced for ABCD3 accumulate VLCFA and express proinflammatory mediators suggesting that dysfunction of those transporters induces proinflammatory signaling pathways in these cell types. Further, macrophages (65) from ALD mouse and human X-ALD-derived lymphoblasts (58) were also reported to produce higher levels of inflammatory mediators as compared to the respective control cells. These studies document that cells induced to accumulate VLCFA become prone to higher expression and release of inflammatory mediators. But there are still very many unknowns as similar cell types defective for ABCD1 function, in ALD mouse brain, do not produce these inflammatory mediators.

In cALD, the inflammation affects the white matter and related cells (45,49). In addition the neuropathology of cALD is associated with the infiltration of vascular inflammatory immune cells (22,48) and NO-mediated toxicity as evident from the detection of nitrotyrosine in the brain of X-ALD patients (21). Myelin breakdown products, as a result of myelin instability, containing complex lipids and/or acylated proteins enriched in VLCFA were believed to play a role in this process possibly by inducing CNS resident immune cells to produce cytokines/chemokines that further upregulate the cellular events to induce myelin

breakdown (1,10). However, recent studies with ABC peroxisomal transporter (ABCD1 and/or ABCD2) silenced astrocytes and C6 glial cells silenced for ABCD3 in culture indicate that metabolic derangements, without myelin degradation products, are sufficient to induce inflammatory response (56). The observed expression of proinflammatory cytokines such as TNF- α , IL-1 β , and IFN- γ in X-ALD brain indicates a role for these cytokines in inflammatory demyelination (38,48). Studies using a combination of super gene array, RT-PCR, and immunohistochemistry techniques have demonstrated the presence of cytokines (IL-1beta, TNF-alpha, IL-6, IL-2, IL-3, and GM-CSF), chemokines (MCP-1, MCP-3, MIP-1b, SDF-2, fractalkine, eotaxin, MIP-2, MDC, HCC-4), chemokine receptors (CCR-1, CCR-4, CCR-5) and iNOS in regions of cALD brains such as the demyelinating plaque and the active edge of the demyelinating plaque compared with histologically unaffected areas of X-ALD brain and appropriate age-matched control brains (42). Brain tissue in the inflammatory area had higher levels of proinflammatory cytokines (eg, TNF alpha, IL-1 β , IL-6, IL-2) and chemokines (eg, MCP-1, MCP-3, SDF-2). However, histologically normal-looking areas of cALD brain when compared with control brain also had increased levels of proinflammatory cytokines (TNF-alpha, IL-1 β , IL-6, IL-2) and chemokines (CCL-2,4,7,11,16,21 and -22 and CC-1,2,4,5), indicating a relationship between metabolic abnormality and inflammatory disease in the absence of vascular infiltrates (42).

As suggested by recent studies, the increased oxidative stress/damage (17,50) and reduction in plasmalogens (5,33) participate in the transition of VLCFA-mediated metabolic disease into neuroinflammatory disease. These conclusions are supported by the following observations: (i) loss of ABCD1 function in cultured astrocytes (56) and in the X-ALD mouse lead to excessive accumulation of VLCFA and increased oxidative stress/damage (17,46,52); (ii) red blood cells and white matter samples of X-ALD patients contain decreased levels of plasmalogens (5,33,39,64); (iii) and neither ABCD1 null mice nor mice deficient in plasmalogen synthesis develop cerebral demyelination whereas the double mutant do develop microgliosis and moderate myelin abnormalities in CNS (5). These observations indicate that derangements in VLCFA and plasmalogens induce oxidative damage leading to expression of lipolytic enzymes and their products (34) followed by enhanced expression of proinflammatory mediators (33,56). However, interpretation of studies from mouse mutants lacking plasmalogens deserve some caution as plasmalogen deficiencies have also been described in other neuroinflammatory disorders. Delineation of molecular events required for transition of VLCFA-induced oxidative stress/damage into neuroinflammatory disease is an active area of investigation. Because over 30% of patients with AMN transition into cerebral ALD, analysis of tissues from these patients may provide clues to events associated with transition of metabolic disease into the neuroinflammatory disease of cALD.

The Third Hit: Generalized Loss of Peroxisomal Function and Neurodegeneration in cALD

The similarities in the neuropathology in the animal model of global peroxisomal dysfunction in oligodendrocytes (the Pex 5 null mice) compared with the double ABCD1/Pex 7 null or to the double ABCD1/ABCD2 null mice suggest that generalized loss of peroxisomal functions may participate in cALD disease pathology (5,30). Pex 5 and Pex 7 (peroxines) as receptors for peroxisome targeted proteins (PTS1 and PTS2), respectively, play pivotal role in the biogenesis and maintenance of peroxisomes (62).

The inflammatory mediator-induced downregulation of function of peroxisomes in hepatocytes (3), liver (6), C6 glial cells (31) and CNS of EAE, an animal model of MS (55), indicate a role for decreased peroxisomes in the pathobiology of neuroinflammatory disease of cALD. Second, the cytokine-mediated compromised peroxisomal integrity, and impaired

peroxisomal functions (3,6,32) indicate for overall negative consequences for myelin synthesis/repair processes. Further, observed higher levels of VLCFA in demyelinating plaque and demyelinating inflammatory region compared with histologically normal-looking brain region of cALD brain indicate that peroxisomal functions were altered by the inflammatory mediators (42). Accordingly, cytokine treatment of cultured cells compromised the VLCFA β -oxidation and other peroxisomal functions (31,42). Therefore, possibly the decrease in peroxisomes and peroxisomal functions (eg, VLC fatty acids β -oxidation, plasmalogen and docosahexaenoic acid biosyntheses) in inflammatory regions of X-ALD brain will further increase the accumulation of VLCFA (31), thus amplifying the inflammatory response (production of cytokines) in brain tissues. As expected, the plaque and inflammatory areas of cALD brain had 2.5- and 3.8-fold greater VLCFA levels compared with histologically normal-looking areas in cALD brain (31,42). Indeed, studies from our laboratory using cultured C6 cells demonstrated that cytokine (TNF- α , IL-1 β , INF- γ) treatment of these cells negatively affect the metabolism of VLCFA such as inhibiting their oxidation and thereby accumulation of these fatty acids (31). By using inhibitors of nitric oxide synthase, NO was identified as one of the intermediates in this inhibition (9). These observations indicate that in addition to downregulation of peroxisomes/proteins, the inflammatory mediators (cytokines) induce inactivation of peroxisomal activities via NO-mediated mechanisms. Further, the downregulation of peroxisomal proteins/biogenesis under inflammatory disease conditions is supported by the observed decrease in peroxisomes/protein in the liver of rats treated with sublethal doses of endotoxin, a Peroxisome proliferator activated receptor alpha (PPAR α)-dependent mechanism (6,43). A sublethal injection of bacterial endotoxin produced drastic changes in peroxisomal structure and function. Structural changes were characterized by alternation in membrane lipid composition and hence the fluidity properties (6,32). Changes in function were associated with a decrease in β -oxidation of VLCFA. Overall, there was a marked decrease in the population of peroxisomes of normal density (6). These observations indicate a generalized loss of peroxisomes and thereby their functions in the inflammatory regions of CNS suggesting that a generalized loss of peroxisomal function participates in X-ALD neuropathology. Accordingly, mice in which most peroxisome functions are selectively disrupted by conditional knockout of Pex 5 in oligodendrocytes exhibited not only an accumulation of VLCFA and deficiency of plasmalogens but also modeled the entire neuropathological phenotype of human X-ALD including axonal degeneration, cerebral demyelination and neuroinflammation (30). Recent report showing deficiency of peroxisomes/peroxisomal dysfunction in neuroinflammatory disease models of EAE, Krabbe disease and cerebral palsy indicate that in addition to cALD and other peroxisomal disorders, inflammatory-compromised peroxisomal functions may also participate in the pathobiologies in these disease conditions (56).

Concluding Remarks

In light of recently generated evidences, we propose a three-hit hypothesis as a framework to study the disease pathogenesis of X-ALD. The metabolic derangements characterized by excess of VLCFA and lower plasmalogens levels and oxidative stress (first hit), which in turn, generates inflammatory disease (second hit) with the participation of environmental, stochastic, genetic or epigenetic factors. Subsequently, the cytokine and chemokine mediators (inflammatory response) further cause a generalized loss of peroxisomes/peroxisomal functions (third hit), creating a vicious circle resulting in cell loss and progressive inflammatory demyelinating disease. Therefore, therapy for X-ALD inflammatory disease should also be considered while developing treatment strategies for the metabolic and oxidative stress disease aspects of X-ALD. Pathophysiological events of ABCD1 dysfunction may vary in different cell types (eg, astrocytes/microglia vs. oligodendrocytes vs. neurons) of CNS and are likely to be dependent on the relative

distribution of peroxisomal ABC transporters and cellular metabolic needs for VLCFA by different cell types. Elucidation of cell autonomous vs. noncell autonomous mechanisms are needed to direct future cell therapeutic approaches.

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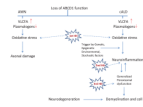


Figure 1.
Three-hit hypothesis on the physiopathogenesis of X-ALD.