ABC Subfamily D Proteins and Very Long Chain Fatty Acid Metabolism as Novel Targets in Adrenoleukodystrophy

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Abstract: Peroxisomes are involved in a variety of metabolic processes, including β-oxidation of fatty acids, especially very long chain fatty acids. Three peroxisomal ABC proteins belonging to subfamily D have been identified in mammalian peroxisomes that have an important role in fatty acid metabolism. ABCD1/ALDP and ABCD2/ALDRP are suggested to be involved in the transport of very long chain acyl-CoA, and ABCD3/PMP70 is involved in the transport of long chain acyl-CoA. ABCD1 is known to be responsible for X-linked adrenoleukodystrophy (X-ALD); an inborn error of peroxisomal β-oxidation of very long chain fatty acids. X-ALD is characterized biochemically by the accumulation of very long chain fatty acids in all tissues, including the brain white matter. Progressive demyelination of the central nervous system and adrenal dysfunction have been observed. The pharmacological up-regulation of peroxisomal β-oxidation of very long chain fatty acids and the suppression of fatty acid elongation are important aspects of an optimal therapeutic approach. Attractive targets for the treatment of X-ALD patients include the ABCD2 as well as elongase that is involved in the elongation of very long chain fatty acids. In addition, stabilization of mutant ABCD1 that has retained some of its function might be another approach, since most of the mutant ABCD1s with a missense mutation are degraded rapidly by proteasomes before or after targeting to peroxisomes. Protection of the central nervous system against oxidative damage is also important in order to delay the progress of disease. We summarize recent pharmaceutical studies and consider the potential for future X-ALD therapies.

Keywords: ABC protein, adrenoleukodystrophy, fatty acid β-oxidation, neurodegeneration, peroxisome, very long chain fatty acids.

INTRODUCTION

Peroxisomes are organelles bounded by a single membrane that are present in almost all eukaryotic cells. These organelles are involved in a variety of metabolic processes, including the β-oxidation of fatty acids, especially very long chain fatty acids (VLCFA), and the synthesis of ether phospholipids and bile acids in mammals [1]. These metabolic pathways require the transport of metabolites in and out of peroxisomes [2]. Recently it has become clear that the transport of such metabolites is facilitated by at least several different metabolic transporters. One of the transporter families is the ATP-binding cassette (ABC) protein. They are a superfamily of membrane-bound proteins whose structure is highly conserved from eubacteria to mammals and which catalyze the ATP-dependent transmembrane transport of a wide variety of substrates, including lipids.

To date, three ABC proteins classified into "subfamily D" have been identified in mammalian peroxisomes. These are adrenoleukodystrophy protein (ALDP/ABCD1), ALDP-related protein (ALDRP/ABCD2), and a 70-kDa peroxisomal membrane protein (PMP70/ABCD3) [3-8]. Dysfunction of ABCD1 is the cause of the human genetic disorder, X-linked adrenoleukodystrophy (X-ALD), which is characterized by an accumulation of VLCFA because of an impaired peroxisomal β-oxidation of VLCFA. VLCFA β-oxidation in X-ALD patient fibroblasts was restored by the expression of ABCD1 [9-11]. Likewise, the expression of ABCD2, which has a high sequence similarity to ABCD1, also restored VLCFA β-oxidation in X-ALD fibroblasts [12]. These data indicate that ABCD1 and ABCD2 are involved in the metabolic transport of VLCFA. ABCD3 is suggested to be involved in the metabolic transport of long chain fatty acids, since the overexpression of ABCD3 in CHO cells induced the β-oxidation of palmitic acid [13]. *Abcd3*(-/-) knockout mice exhibited abnormalities in peroxisomal metabolism of the bile acid intermediates, pristanic acid and phytanic acid, suggesting that ABCD3 is involved in the transport of bile acid intermediates and branched chain fatty acids (Jimenez-Sanchez *et al. Am J Hum Genet* 2000, meeting abstract). ABCD3-related protein (P70R/ABCD4) is also a member of ABC protein subfamily D [5, 14]. Recently, we found that ABCD4 is localized to the endoplasmic reticulum (ER), not to peroxisomes, but the function of ABCD4 still remains unknown [15].

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1389-4501/11 $58.00+.00

X-ALD is caused by mutations in the ABCD1 gene [3] and affected patients show progressive demyelination in the central nervous system (CNS), adrenal insufficiency, and testicular dysfunction as pathological characteristics. Severe forms of X-ALD are associated with inflammatory demyelinative lesions. Lymphocytes, reactive astrocytes and macrophages are suggested to be involved in the formation of these lesions. The pathological features of X-ALD may be accounted for by the overabundance of VLCFA, and the abnormal accumulation of VLCFA is the most likely culprit behind the initiation and progression of the disease. However, the molecular basis of the disease is not well understood.

The therapeutic options for X-ALD are limited at present. Lorenzo's oil, a 4:1 mixture of glycerol triolein and glycerol trierucate, normalizes VLCFA levels in the plasma of X-ALD patient, but it does not alter the clinical progress of patients with neurological symptoms [16, 17]. Hematopoietic stem cell transplantation (HSCT) is currently the only effective treatment for X-ALD patients with the childhood cerebral form. However, the availability of HSCT is limited by compatible donors, and HSCT carries a high mortality risk from complications. In addition, HSCT is known to be effective only for asymptomatic patients and patients with the early stage of cerebral demyelination [18]. Under such circumstances, alternative therapeutic approaches based on biochemical and pathological characteristics of the disease obviously need to be developed. The pharmacological induction of *ABCD2* gene expression, stabilization of mutant ABCD1 with proper functionality, stimulation of VLCFA β-oxidation, inhibition of elongase, which is involved in VLCFA synthesis, and suppression of inflammatory events in glial cells, are all potentially important strategies in the treatment of X-ALD.

STRUCTURE AND FUNCTION OF ABCD1

Structure of ABCD1

ABCD1 has the predicted structure of a half-size ABC protein with one transmembrane domain (TMD) and one nucleotide-binding domain (NBD). The hydropathy profile of human ABCD1 predicts that the amino terminal half of ABCD1 is hydrophobic with six transmembrane segments, and the COOH-terminal half is hydrophilic, having NBD (Fig. **1A**) [19]. Protease treatment of peroxisomes indicated that the NBD of ABCD1 is exposed to the cytosol [19]. As

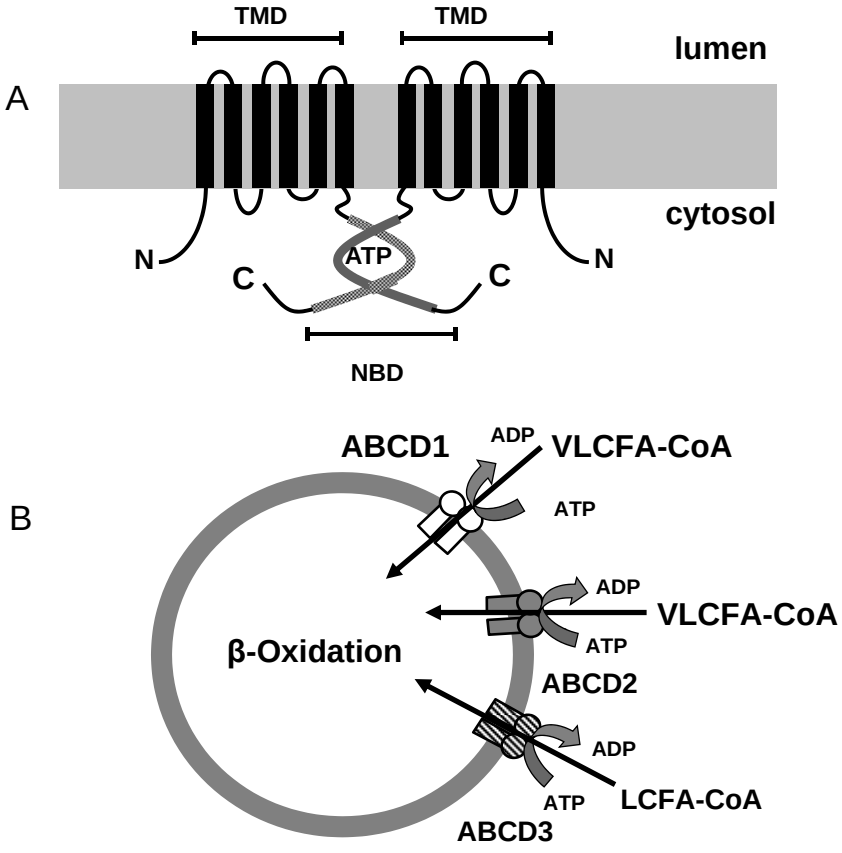

Fig. (1). The putative secondary structure of ABCD1 and function of peroxisomal ABC proteins. A, Six TMDs are located in the N-terminal half of the protein, and a NBD including the Walker A, B and ABC signature sequences are located in the C-terminal half of the protein. ABCD1 is a half-size ABC protein and predominantly exists as a homodimer. **B**, In mammals, three ABC proteins belonging to the D subfamily are known to exist in peroxisomes, ABCD1, ABCD2, and ABCD3. Among them, ABCD1 and ABCD2 have been suggested to be involved in the transport of very long chain acyl-CoA (VLCFA-CoA) from the cytoplasm into the peroxisomes. ABCD3 is thought to be involved in the transport of long chain acyl-CoA (LCFA-CoA).

for the ATP-binding and hydrolysis activities of ABCD1, Roerig *et al*. reported that the recombinant NBD of ABCD1 bound and hydrolyzed ATP [20]. Tanaka *et al.* also detected the ATP binding and hydrolysis activities of native ABCD1 in rat liver peroxisomes by photoaffinity labeling with 8-azido-[α-^{32}P]ATP and 8-azido-[γ-^{32}P]ATP [21]. On the other hand, the TMD of ABCD1 is proposed to be involved in substrate-recognition and to form a transport pathway across peroxisomal membranes. Guimarães *et al.* assessed the substrate-induced conformational alterations in ABCD1 with a protease-based assay, and found that long- and very long chain acyl-CoA increased the sensitivity of the NH_2-terminal 44-kDa fragment of ABCD1 to Factor Xa, and this acyl-CoA induced sensitivity was reversed by the presence of ATP-γS [22]. These findings suggest that the NH_2-terminal TMD of ABCD1 is involved in the recognition of these substrates and undergoes a conformational change upon ATP binding to the COOH-terminal NBD of ABCD1.

As most of the half-size ABC proteins identified to date dimerize to form a functional transporter, it has been suggested that the peroxisomal ABC proteins also need to assemble as homo- or heterodimers on the peroxisomal membranes to form a functional unit. As for the quaternary structures of peroxisomal ABC proteins, Liu *et al*. were the first to show the occurrence of homo- as well as heterodimeric interactions among ABCD1, ABCD2, and ABCD3 by using a yeast two-hybrid system and co-immunoprecipitation experiments [23]. We also showed by means of co-immunoprecipitation studies that ABCD1 forms a stable complex with ABCD3 and certain peroxisomal proteins on rat liver peroxisomal membranes [21]. On the other hand, Guimarães *et al*. reported that mouse liver ABCD1 was a mostly homomeric protein assembly, based on sucrose density gradient analysis and immunoprecipitation experiments with digitonin-solubilized mouse liver peroxisomes [24]. Furthermore, FRET microscopy experiments in intact living cells demonstrated that ABCD1 predominately forms a homodimer, although ABCD1 can form a heterodimer with ABCD3 [25]. These data suggest that ABCD1 mainly exists as a homodimer in mammalian peroxisomal membranes, although ABCD1 can form a complex with ABCD2 and ABCD3, and is involved in the ATP-utilizing transport of CoA derivatives of VLCFA.

Function of ABCD1

The biochemical hallmark of X-ALD is an impaired oxidation and accumulation of saturated VLCFA in cerebral white matter, adrenal glands, fibroblasts, and plasma [26]. Furthermore, *Abcd1*(-/-) mice also displayed the accumulation of VLCFA in tissues [27-29]. Although the precise mechanism by which ABCD1 is involved in fatty acid metabolism still remains to be elucidated, these pathognomonic characterizations of X-ALD allow us to deduce the function of ABCD1 to be a very long chain acyl-CoA transporter on peroxisomal membranes. Transfection of *ABCD1* cDNA into X-ALD skin fibroblasts restored the β-oxidation of lingoceric acid, and consequently, the VLCFA content returned to normal in the fibroblasts [9-11]. The expression levels of ABCD1 were correlated to VLCFA β-oxidation activities in primary and SV40T-transformed human skin fibroblasts [30]. Recently, van Roermund *et al.* showed that ABCD1 can function as a homodimer and is involved in the transport of a range of substrates including palmitic, oleic, behenic, and tetracosahexaenoic acid across the peroxisomal membranes by means of the expression of the human *ABCD1* cDNA in yeast *Saccharomyces cerevisiae* [31]. These findings strongly suggest that ABCD1 is involved in the uptake of activated VLCFAs into mammalian peroxisomes.

A variety of genetic and biochemical studies on ABCD1-related peroxisomal ABC proteins from other organisms have led to the same conclusion. For example, disruption of *PXA1* and/or *PXA2*, the only two peroxisomal ABC proteins known in *Saccharomyces cerevisiae*, resulted in impaired growth of these mutants on oleic acid as a sole carbon source, and a reduced ability to oxidize oleate [32-34]. Furthermore, Verleur *et al.* showed that Pxa2p is directly responsible for the ATP-dependent transport of long-chain acyl-CoA across peroxisomal membranes by using a semi-intact yeast cell system [35]. Recently, a similar role was proposed for a plant ABCD1 homologue, as well. In *Arabidopsis thaliana* the gene known variously as *PXA1*, *PED3*, or *CTS* encodes a full-size ABC protein mainly referred to as COMATOSE. Both halves of COMATOSE showed significant sequence identity to the human ABCD1. Zolman *et al.* reported that the *Arabidopsis PXA1* mutant grew slowly compared with wild type, with smaller rosettes, fewer leaves, and shorter inflorescence stems under hormone-free medium with auxin indole-3-butyric acid (IBA) [36]. IBA is converted to the more active auxin indole-3-acetic acid by peroxisomal β-oxidation, suggesting that the *PXA1* mutant has a defect in the import of IBA into peroxisomes. Recently, the loss of *CTS* in *Arabidopsis thaliana* exhibited a severe deficit in the breakdown of lipid bodies in germinated cotyledons [37]. In the *CTS* mutant, C20 and C22 acyl-CoA, which are predominantly derived from triacylglycerol, accumulated in seeds and seedlings. Furthermore, the *Arabidopsis PXA1* mutant under prolonged dark conditions exhibited an accumulation of free fatty acids, including palmitic acid, 7,10,13-hexadecatrienoic acid, and, α-linolenic acid, in mature leaves [38]. These findings also indicate that ABCD1 is involved in the uptake of activated VLCFAs across peroxisomal membranes.

MUTATION OF ABCD1 AND ADRENOLEUKODYSTROPHY

Mutations in ABCD1

The gene that is defective in X-ALD was mapped to Xq28 [39] and isolated and cloned by Aubourg and his colleagues [3]. The gene referred to as *ABCD1* is composed of 10 exons, and it codes for an mRNA of 4.3 kb, with a protein of 745 amino acids that is referred to as ABCD1. Thus far, more than 500 mutations widely distributed over the *ABCD1* gene have been identified (http://www.x-ald.nl). Missense mutations comprise ~60% of all of the mutations. The mutations have been found throughout the entire gene, although there is a clustering of mutations in the NH_2-terminal half of ABCD1, including TMD1-6, loop1-5 (40%) and NBD (30%) [40]. We have been analyzing the mutations of ABCD1 in Japanese probands with X-ALD and their families, and identified 55 mutations in 63 Japanese X-ALD kindreds, which included 35 missense mutations, 5 nonsense

mutations, 7 frame shift mutations, 3 amino acids deletions, 2 exon skip mutations and 3 large deletions

Among the missense mutation in X-ALD patients, it has been reported that ~50% of mutant ABCD1s were not detected and ~15% of them were reduced in amount in X-ALD fibroblasts, based on immunofluorescent or immunoblot analysis. Recently, we showed that some mutant ABCD1s were degraded by proteasomes or additional protease(s) before or after transport to peroxisomes [41].

Clinical Symptoms

There are various clinical phenotypes, such as the childhood cerebral form with cerebral demyelination and childhood onset (CCALD), the adolescent cerebral form (AdoCALD), the adult cerebral form (ACALD), adrenomyeloneuropathy (AMN) with axonopathy of the pyramidal and somatosensory tracts along with peripheral neuropathy, the olivo-ponto-cerebellar form (OPC), and Addison's disease alone [42]. We performed a retrospective nation-wide epidemiological survey of X-ALD in Japan during the 1990s [43]. However, no consistent correlation between phenotype and either specific mutations or ABCD1 expression was documented. The existence of modifier genes has been postulated [44].

CCALD is the most common phenotype, and is characterized by a progression of intellectual, psychological, visual and gait disturbances which first appear during the period school age. Patients are often misdiagnosed as having attention-deficit hyperactivity disorder, psychological problems, or either ophthalmic or ear abnormalities at the onset of the initial symptoms. Therefore, quite a number of patients are not diagnosed until further symptoms manifest, such as seizures, gait disturbances and/or other neurological symptoms. Brain MRI findings characteristically show an enhanced T2 signal, even at the early stage of the disease, suggesting extensive demyelination has occurred. It is hypothesized that the demyelination is associated with the cerebral inflammation. The prognosis is generally very poor and patients are considered likely to die within a few years, although recently good general care has apparently improved this unfavorable prognosis.

AdoCALD is similar to CCALD, but the appearance of symptoms occurs later than in CCALD, and develop more slowly. The age of onset is 11-21 years. ACALD is characterized by a slower progression of psychological symptoms, is more common in Japan than in Western countries [43], and is sometimes misdiagnosed as dementia or a psychological disorder. Most AMN patients manifest slowly progressive gait disturbances as the initial symptom, while sensory and autonomic disturbances occur in some patients. The mean age of onset of AMN was reported to be 30.2 (13-51) years in Japan [43], and about half of these patients exhibited cerebral involvement approximately 10 years after onset. OPC is characterized by cerebellar ataxia and pyramidal tract involvement, manifest gait disturbance as the initial symptom, and cerebellar ataxia which becomes evident several months to 1 year after onset. Approximately half of all OPC patients also display cerebral involvement, with concomitant symptoms such as intellectual and psychological problems [45]. This OPC form has been reported predominantly in Japan [46]. Patients with Addison's disease alone manifest adrenal insufficiency, including unexplained vomiting and weakness or coma, with first appearance occurring somewhere between childhood and adulthood.

Pre- or asymptomatic patients will progress to the various phenotypes described above between 3 and 50 years old. Therefore, it is important to provide information to patients and family members by means of genetic counseling. Diagnosis of pre-symptomatic boys before 3 years old, and long-term follow-up using subtle neuropsychological signs, brain MRI, electrophysiological investigation, adrenal function tests, and HSCT are of benefit. About half of the female carriers over the age of 40 years will develop mild to severe neurological symptoms caused by the spinal cord and peripheral nerve abnormalities, such as weakness and spasticity of the legs, impaired sensation of the lower limbs and autonomic disturbances, similar to AMN.

Pathogenesis of X-ALD

Mutations in the *ABCD1* gene result in increased VLCFA in tissues and body fluids of patients with X-ALD, and reduced VLCFA β-oxidation in peroxisomes. Impaired transport of very long chain acyl-CoA by ABCD1 explains, in part, the biochemical defects. Recently Ofman *et al.* have shown that a deficiency of ABCD1 raised cytosolic levels of very long chain acyl-CoAs in X-ALD fibroblasts and the substrates were then further elongated by the enzyme called elongation of very long chain fatty acids-1 (ELOVL1), which catalyzes the synthesis of both saturated (C26:0) and mono-unsaturated VLCFA (C26:1) [47]. In addition, knockdown of ELOVL1 reduced the elongation of C22:0 to C26:0 and lowered C26:0 levels in X-ALD fibroblasts, suggesting that ELOVL1, in addition to ABCD1, is important for the accumulation of VLCFA.

The mechanism by which the accumulation of VLCFA in the brain causes neurodegeneration, especially demyelination, remains obscure. However, the association of VLCFA accumulation with the immunoresponse is thought to be important (Fig. **2**). It has been demonstrated that VLCFA accumulation subsequently leads to a neuroinflammatory response, with the production of proinflmmatory cytokines by activated astrocytes and microgial cells together with demyelination and a loss of oligodendrocytes [48]. Direct toxicity of VLCFA has been demonstrated to result from an alteration of membrane fluidity in erythrocytes [49]. Recently, Hein *et al.* have shown a direct toxic effect of C26:0 on primary neurons and glial cells, especially oligodendrocytes, via mitochondrial dysfunction and Ca^{2+} deregulation [50]. The presence of less mature myelin in weaning and postweaning rats treated with hexacosanoic acid [51], suggests that an increase of VLCFA may lead to myelin instability, followed by an inflammatory or immune-mediated process, and therefore contribute, at least in part, to the loss of oligodendrocytes observed in the plaques of X-ALD brains. Moreover, the down-regulation of peroxisomal VLCFA β-oxidation may lead to a decrease of docosahexaenoic acid (DHA) synthesis. DHA is not directly incurporated into the myelin sheath, but its presence is crucial for oligodendrocyte maturation and the formation of mature myelin. The complex lipids of VLCFAs or VLCFA-phospholipids could serve as an antigen recognized by the

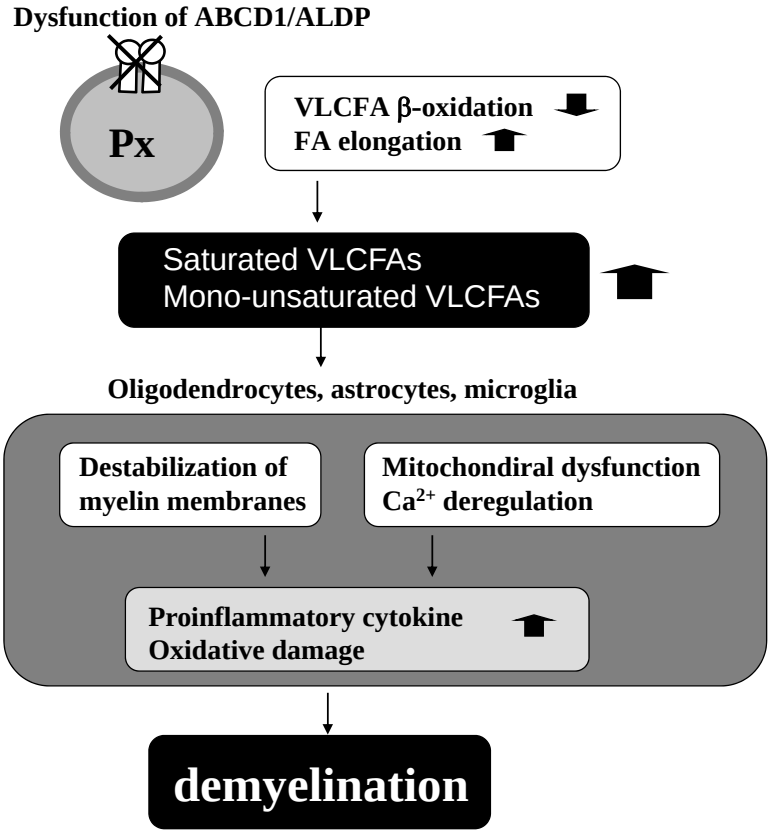

Fig. (2). A putative mechanism of neurodegeneration in X-ALD. In the CNS, abnormal accumulation of VLCFAs, which is caused by the dysfunction of ABCD1 followed by a reduction in peroxisomal VLCFA β-oxidation and/or an induction of fatty acid elongation, might result in the destabilization of myelin membranes and/or mitochondrial dysfunction. The myelin membrane fragments activate the astrocytes and/or microglia, which event leads to the inflammatory reaction. Proinflammatory cytokines are known to have negative affects on oligodendrocytes. Alternatively, an excess of VLCFA might adversely affect mitochondria, which would exacerbate oxidative stress in oligodendrocytes.

CD1 pathway, which would also be a plausible candidate to trigger inflammatory demyelination [52]. In addition, there is increasing evidence that oxidative stress contributes to the pathogenesis of the cerebral inflammatory phenotype, and possibly AMN. Gilg *et al*. demonstrated increased levels of inducible nitrous oxide synthase, and the presence of nitrosylated proteins in astrocytes and macrophages, in affected post-mortem tissue [53]. Powers *et al*. found convincing evidence of oxidative stress (increased levels of hemoxygenase 1 and manganese–superoxide dismutase) and oxidative damage from lipid peroxidation (4-hydroxynonal and malondialdehyde), as well as nitrosylated proteins, in the post-mortem brain tissue of four X-ALD patients with the cerebral inflammatory phenotype [54].

Diagnosis

The definitive diagnosis of X-ALD, as well as female carrier detection is achieved by demonstration of the biochemical defect along with mutation analysis of the *ABCD1* gene. An elevated VLCFA confirms the diagnosis of both X-ALD and the carrier, but there can be overlap of the C24:0/C22:0, C25:0/C22:0 and C26:0/C22:0 ranges between healthy controls and at least 10% of the carriers. Furthermore, 3 to 7 percent of patients with X-ALD are the result of a spontaneous mutation of the *ABCD1* gene, and thus the mothers of these patients are not carriers (hppt://www.x-ald.nl). For these reasons, mutation analysis of the *ABCD1* gene is necessary not only for carrier detection, but also for detecting a spontaneous mutation. Carrier detection is very important to identify pre-symptomatic patients and female carriers within their kindred. Newborn screening may be a potential method for widely identifying pre-symptomatic ALD patients and female carriers. Hubbard *et al.* have undertaken a mass screening pilot study by the detection of 1-hexacosanoyl-2-lyso-sn-3-glycerophosphoryl-choline (26:0-lyso-PC) using a combined liquid chromato-graphy-tandem mass spectrometry (LC-MS/MS) method [55].

Additionally, the establishment of a long-term follow-up system for these pre-symptomatic patients is needed, because we cannot at present predict the timing for HSCT so as to get beneficial effects or achieve a good clinical outcome. In our nationwide survey in Japan, approximately 30% of the X-ALD cases exhibited cerebral symptoms before the age of ten years, but nearly 50% of the patients had no obvious symptoms until they were twenty years old [43]. Thus, further break-throughs in diagnostic and therapeutic

approaches for X-ALD are keenly anticipated. In particular, the identification of predictive factors for the onset of brain involvement is of critical importance.

Therapeutic Approaches

The level of VLCFA does not predict the development of the disease state in X-ALD, and the exact link between abnormal VLCFA accumulation and the pathogenesis is still unclear at present. However, abnormal accumulation of VLCFA in brain is the most likely culprit for the initiation and progression of the disease.

Dietary therapy with Lorenzo's oil is now a commonly accepted treatment for X-ALD patients. Lorenzo's oil does not halt the progression of the cerebral phenotype X-ALD patients who were already symptomatic when the treatment was initiated. Recent reports, however, suggest that Lorenzo's oil might have demonstrated a preventive effect in asymptomatic boys whose brain MRI is normal [56, 57], and preliminary data from dietary therapy with Lorenzo's oil indicates that it seems to reduce the progression of pure AMN [58]. Golovko and Murphy reported that erucic acid (22:1n-9) can cross the blood-brain barrier, and is incurporated into cholesterol esters, triacylglycerols and phospholipids pools as either 22:1n-9 or its chain-shortened metabolites, 20:1n-9 and 18:1n-9, due to the chain shortening of 22:1n-9 [59]. It is thus possible that adverse structural changes in certain lipid classes in the X-ALD brain are attenuated by treatment with Lorenzo's oil, which might help prevent the disease onset. Elucidation of the precise mechanisms of Lorenzo's oil effect in the CNS could provide important clues to finding targets for the initiation of cerebral ALD, such as CCALD.

Lovastatin, an HMG-CoA inhibitor, is reportedly a potential therapeutic drug for X-ALD since it decreases plasma VLCFA in X-ALD patient [60]. However, in a comparable study using lovastatin and simvastatin, the VLCFA content could not be normalized in tissues and plasma of *Abcd1*(-/-) mice [61, 62]. It is hypothesized that lovastatin additionally may have a favorable effect, since it down-regulated the synthesis of NO in X-ALD lymphoblasts along with the decrease in VLCFA levels [63]. However, the

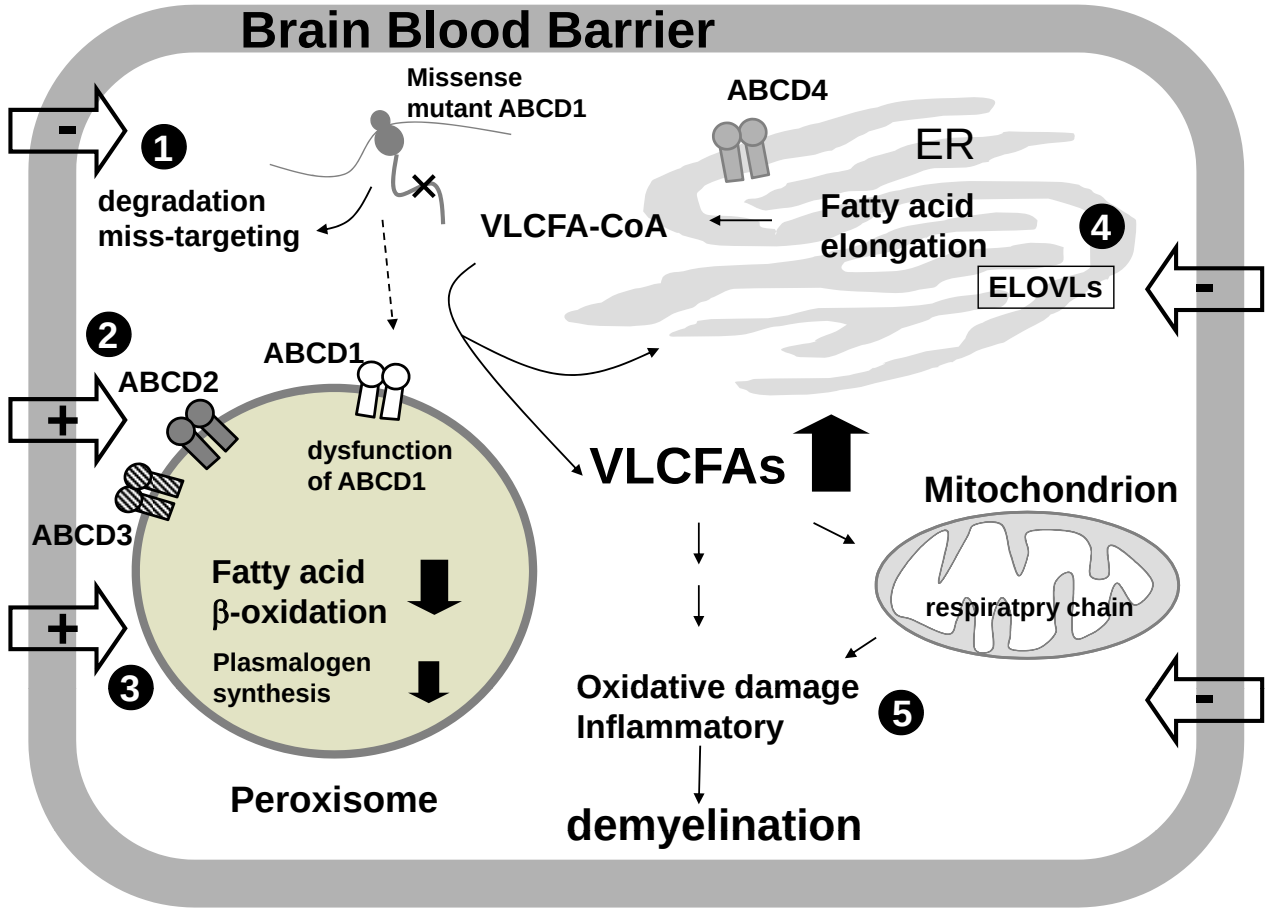

Fig. (3). Possible targets for X-ALD therapy. Mutation of ABCD1 leads to the dysfunction of ABCD1, which results in a decrease in VLCFA β-oxidation. The decrease in VLCFA β -oxidation leads to an increase in the VLCFA-CoA level, which is the substrate for fatty acid elongation in the ER. As a result, the VLCFA level is increased and the subsequent abnormal accumulation of VLCFAs results in oxidative damage. Stabilization and correct subcellular localization of missence mutants of ABCD1 with residual activity might be an effective way to recover the dysfunction of ABCD1 ❶. Induction of *ABCD2* expression ❷, stimulation of peroxisomal fatty acid β-oxidation ❸ or inhibition of microsomal fatty acid elongation ❹ are attractive approaches to reduce excess VLCFA levels. Attenuation of the dysregulated response to oxidative stress also has potential as an effective approach in X-ALD ❺. Chemical compounds that can pass through brain-blood barrier and act on these targets would be promising drugs for X-ALD therapy.

clinical efficacy of lovastatin has not yet been clarified. Very recently Engelen *et al.* demonstrated lovastatin leads only to a small decrease of plasma C24:0 and C26:0 in X-ALD patients by a nonspecific result of the decrease in the level of LDL cholesterol and they indicated that lovastatin should not be prescribed as a therapy to lower levels of VLCFA in patients with X-ALD [64]. In the following sections, we will focus our attention on other attractive X-ALD therapeutic targets over the past few years (Fig. **3**).

ABCD2 as a Target Molecule

Among the members of ABC protein subfamily D, ABCD2 shows the highest similarity (88%) to ABCD1. Overexpression of ABCD2 prevents the accumulation of saturated VLCFA in the adrenal gland and brain, and neurological signs of disease, which are observed in *Abcd1*(-/-) mice [65]. In addition, the X-ALD phenotype is independent of the *ABCD2* genotype, and *ABCD2* can be excluded as a modifier locus for clinical diversity in X-ALD [66]. Taken together, the pharmacological induction of expression is a reasonable therapeutic strategy for X-ALD.

ABCD2 transcription is regulated by nuclear factors such as the peroxisome proliferator-activated receptor (PPARα), retinoid X receptor (RXR), thyroid hormone receptor (TRβ) and sterol regulatory element (SRE) binding proteins (SREBP1a, SERBP1c and SREBP2). The SRE located in the *ABCD2* promoter overlaps with a direct repeat separated by 4 nucleotides (DR-4), suggesting cross talk between SREBPs and liver X receptor α (LXRα) or TRβ, which are known to be dimerized with RXRα [67]. Weinhofer *et al.* demonstrated that depression of the cholesterol content results in a decrease in the C26:0 level, because the expression of *ABCD2* gene is up-regulated via the activation of the SREBPs [68, 69]. In addition, the *ABCD2* promoter contains a functional thyroid hormone response element (TRE) by which thyroid hormone can induce the *ABCD2* gene both *in vitro* and *in vivo*. Recently, it was shown that the ligand-activated thyroid hormone receptors TRα and TRβ stimulate or derepress, respectively, the SREBP1-dependent induction of the *ABCD2* promoter [67]. These thyroid hormone receptors bind the SRE/DR-4 motif. Therefore, novel tissue-specific ligands for TRα, TRβ, or other DR-4 binding factors that interact with SREBP1, might enhance *ABCD2* expression in the brain. Recently, Genin *et al.* have reported that *ABCD2* gene is induced by the administration of halogen-free thyromimetics (GC-1 and CGS 23425) specific for TRβ receptors in *Abcd1*(-/-) mice. GC-1 was shown to be able to enter the brain and thus might be effective to induce gene expression, especially in oligodendrocytes. Therefore, these thyromimetics specific for TRβ may have the capacity to induce *ABCD2* genes in the brain [70] and therefore, could be attractive candidates for X-ALD therapy.

Dehydroepiandrosterone (DHEA), the most abundant steroid in humans, was reported to be a novel inducer of the *ABCD2* gene. Gueugnon *et al.* reported that DHEA directly induced *Abcd2* expression and that short-term treatment of mice with DHEA led to the induction of *Abcd2* expression independently of PPARα. Although the induction was only reportedly detected in the liver in their experiments, the long-term administration of DHEA may be beneficial for X-ALD patients [71].

Concerning the induction of *ABCD2*, 4-phenylbutyrate (4-PBA) and valproic acid, which are categorized as histone deacetylase (HDAC) inhibitors [72] , are potential candidates for the treatment of X-ALD. 4-PBA crosses the blood-brain barrier, and the administration of 4-PBA to *Abcd1*(-/-) mice resulted in the decrease in VLCFA level in the brain and adrenal glands via the induction of *ABCD2* expression or the activation of mitochondrial fatty acid β-oxidation [73]. However, this drug is very rapidly metabolized in the liver and has a very short half-life *in vivo*, indicating that the dosage required for a biological effect in humans makes it unpractical for clinical applications. Actually 4-PBA did not result in a decreased VLCFA levels in a small clinical trial with AMN patients. Therefore, structural analogs of 4-PBA with a longer half-life *in vivo* would be a more attractive candidate for X-ALD therapy [74]. Recently, Fourcade *et al.* have reported that valproic acid induced the expression of the *ABCD2* gene, reduced the level of monounsaturated VLCFA (C26:1) and induced antioxidant effects *in vivo* and *ex vivo* experiments [75]. Since valproic acid is known to be able to cross the blood-brain barrier and is already considered a very safe drug, valproic acid is a promising candidate for X-ALD.

The differences in the expression patterns and phenotypes of *Abcd1*(-/-) and *Abcd2*(-/-) mice suggest other specific roles for ABCD2 in lipid metabolism. Engelen *et al.* reported that cholesterol-deprivation led to the reduction of the C26:0 level along with the increase in *ABCD2* expression in X-ALD fibroblasts [76]. However, the depression of cholesterol resulted in an increased expression of stearoyl-CoA desaturase and increased mono-unsaturated VLCFA level, but did not show any increase in [1-^{14}C]C26:0 β-oxidation. Taken together, ABCD2 may be involved in other forms of metabolism than just C26:0 β-oxidation [76].

Leclercq *et al.* have reported that *ABCD2* expression in the liver is higher in n-3 polyunsaturated fatty acid (PUFA)-deficient rats than rats fed α-linoleic acid or docosahexaenoic acid, presumably because of feedback regulation. They speculated that ABCD2 is involved in the transport of specific classes of fatty acids related to PUFA metabolism into peroxisomes [77]. Fourcade *et al*. have also suggested that ABCD2 might play a role in the transport of DHA or docosapentaenoic acid (DPA) precursors (C24:5n-6 and C24:6n-3) and monounsaturated VLCFAs. They suggested that monounsaturated VLCFAs (C26:1) might be involved in oxidative damage to proteins [78]. These results are consistent with the report by Powers *et al.* that in *Abcd2*(-/-) mice, the oxidative stress in adrenal cells was grater than that in *Abcd1*(-/-) mice, suggesting that ABCD2 might be more important for the control of oxidative stress than ABCD1, at least in the adrenal glands [79].

Taken together, ABCD2 appears to have a central role in the metabolism of unsaturated rather than saturated VLCFAs, which pattern involves the synthesis of DHA and oxidative stress. Since DHA is correlated with the incidence of Alzheimer's disease, ABCD2 may serve as a therapeutic target in common human neurodegenerative disorders [78]. Although the precise role of ABCD2 remains to be elucidated, ABCD2 is a most promising target molecule for X-ALD therapy.

ABCD3 and ABCD4 as a Target Molecule

It has been reported that overexpression of ABCD3 restores VLCFA β-oxidation in X-ALD fibroblasts [10]. Furthermore, stimulation of ABCD3 through the activation of PPARα with fenofibrate overcame the peroxisomal β-oxidation defect in the liver of *Abcd1*(-/-) mice [12]. ABCD3 knockdown generated oxidative stress and pro-inflammatory cytokine production in C6 glial cells [80]. These results suggest that a correction of the biochemical defect in X-ALD should be possible by drug-induced overexpression or ectopic expression of ABCD3. Asheuer *et al.* studied the expression of *ABCD1-4* and two VLCFA synthetase genes (*VLCS* and *BG1*) involved in VLCFA metabolism to elucidate the mechanisms underlying the phenotypic variability of X-ALD. Among these genes, the expression of *ABCD4* and *BG1* tends to be decreased with the severity of the disease, acting early in the pathogenesis of X-ALD [81]. Therefore, ABCD3 and ABCD4 might be target molecules for X-ALD therapy. At present, however, approaches to induce ABCD3 and ABCD4 expression are not realistic since there is no report about induction of these expressions in mice and human brain.

Stability of Mutant ABCD1

It has been reported that ~70% of mutant ABCD1 with a missense mutation were either not detected or were reduced in X-ALD fibroblasts. We found that mutant ABCD1s with the missense mutation in the C-terminal half of ABCD1 were degraded by a protein quality control system associated with proteasomes, and ABCD1 mutants within the loop between TMD2 and 3 resulted in a deficiency in peroxisomal targeting [41]. Therefore, in the case where mutant ABCD1 has residual biological activities, stabilizing or correcting the subcellular localization of the mutant ABCD1 could restore its function. In cystic fibrosis and congenital nephritic syndrome of the Finnish type, defective trafficking of the missense mutants of transmembrane conductance regulator (CFTR) and nephrin, respectively, was rescued by a chemical chaperone, such as 4-PBA or flavonoids [82, 83]. Screening of small molecule libraries to stabilize or to correct the subcellular localization of functionally active ABCD1 mutants might be a beneficial approach for some X-ALD patients bearing a missense mutation. However, a very long way seems to be required to develop useful drugs from this point of view.

Stimulation of Peroxisomal β-Oxidation

Fatty acid β-oxidation activity in peroxisomes is thought to depend on the active transport of acyl-CoA across membranes and passive diffusion of free fatty acids within membranes. In the latter case, fatty acids are activated to acyl-CoA by very long chain acyl-CoA synthetases, such as ACSVL1 and ACSVL5, on the luminal side of peroxisomes. Pillia *et al.* demonstrated that free VLCFA diffuses rapidly through the lipid barrier [84]. Therefore, the activation of very long chain acyl-CoA synthetases in peroxisomes appears to be a target for X-ALD therapy. Recently we found that baicalein 5,6,7-trimethylether, a plant flavonoid, has the capacity to attenuate VLCFA metabolism in X-ALD fibroblasts [85]. This flavonoid activated peroxisomal fatty acid β-oxidation regardless of the carbon chain length. The activation appeared to be caused, at least in part, by the up-regulation of peroxisomal *ACSVL1*, but not *ABCD2*. Therefore, we speculated that baicalein 5,6,7-trimethylether activates peroxisomal fatty acid β-oxidation via an ABCD1-independent pathway [86]. The methoxy residues are important for the activity, suggesting the possibility that structural analogs may attain a greater potency. Further studies of flavonoids and chemically modified derivatives should help provide a new, metabolism-based therapeutic approach for X-ALD therapy.

Suppression of Fatty Acid Elongation

Saturated VLCFAs in the body are derived from both the diet and *de novo* synthesis. In contrast, excessive VLCFAs in X-ALD are largely derived from endogenous synthesis through fatty acid elongation in the ER. Reduction of saturated VLCFAs in plasma by Lorenzo's oil is presumably caused by competitive inhibition of the microsomal fatty acid elongation activity [87-89]. It is thus likely that the suppression of VLCFA synthesis provides a new approach for X-ALD.

The fatty acid elongation reaction in ER consists of 4 sequential reactions. In mammals, seven enzymes (ELOVL1-7) have been identified, which are responsible for the first and rate-limiting step in this reaction cycle [90]. High-density assay, a convenient assay method for measuring the elongation of VLCFA using a Unifilter-96 GF/C plate, revealed that ELOVL1, 3 and 6 preferentially elongated the saturated acyl-CoAs, while ELOVL2 and 5 elongated the unsaturated acyl-CoA [91]. Recently, ELOVL7 was shown to be involved in the elongation of saturated acyl-CoAs (C20:0 ~) [92].

Kemp and colleagues reported enhanced VLCFA elongation activity in X-ALD patient fibroblasts and speculated that they do not result from impaired peroxisomal β-oxidation alone, but also because of the additional effect of unchecked chain elongation [93]. As judged from the substrate specificity, ELOVL1 and ELOVL3 would be the most attractive candidate elongases for enhancing fatty acid elongation in X-ALD. Recently, it was reported that ELOVL1 expression is not increased in X-ALD fibroblasts, but the increased cytosolic very long chain acyl-CoA due to the reduction of peroxisomal fatty acid β-oxidation resulted in an increase in further elongation [47]. They also demonstrated that saturated VLCFA are synthesized via the concerted reaction of ELOVL6 and ELOVL1 and the silencing of ELOVL1 expression led to a decrease in C26:0 levels. The expression of *Elovl1* mRNA is ubiquitous in murine tissues, but potent expression is found in the myelinated parts of the CNS [90]. Although the expression of *ELOVL6* gene was reported to be regulated directly by SREBP1c [94], the transcriptional regulation of *ELOVL1* is still unclear. Down-regulation of *ELOVL1* and/or *ELOVL6* expression or identification of a specific inhibitor of ELOVL1 and/or ELOVL6 might be effective for the reduction of VLCFA.

Oxidative Damage

Several groups have proposed that oxidative stress might be involved in the pathogenesis of X-ALD. Although it is

not clear whether it is primary or secondary in the pathogenesis of X-ALD, the protection of the CNS from oxidative stress is suggested to be a useful therapeutic standpoint. Oxidative damage was demonstrated by the increased markers of lipoxidative damage in post mortem brain samples from cerebral X-ALD patients [54]. In *Abcd1*(-/-) mice, oxidative damage in the spinal cord was observed at as early as 3.5 months of age, more than 1 year before the neuropathological signs appear [95]. Deon *et al.* have reported that the total antioxidant defense was decreased in symptomatic but not in asymptomatic X-ALD patients, suggesting that asymptomatic patients might be protected against oxidative stress because of their normally functioning antioxidant defense systems [96, 97]. Taken together, the functional loss of ABCD1 could correlate with a defective antioxidant response, and oxidative damage may be linked to both the initiation and the progression of X-ALD demyelination.

In X-ALD patients, the plasmalogen level in the white matter was reported to be reduced, which might result in a susceptibility to oxidative stress [98]. Recently, it has been reported that plasmalogen functions in the protection of cells from the oxidative damage caused by VLCFA accumulation [99]. In fact, the myelin abnormalities were observed in *Abcd1*(-/-)*/Pex7*(-/-) mice had plasmalogen synthesis defects in addition to VLCFA accumulation. Taken together, it seems likely that disruption of the endogenous antioxidant system leads to reactive oxygen species (ROS) production and myelin membrane lipid oxidation, which in turn results in the inflammatory disease process.

Furthermore, Singh and colleagues have published several reports about the association of oxidative stress with the pathogenesis of X-ALD [63, 98, 100-102]. Lymphocytes from X-ALD tend to synthesize NADPH oxidase-dependent free radicals, nitric oxide and cytokines. Inflammatory cytokine expression and inducible nitrous oxide systems (NOS) were induced in *Abcd1/Abcd2*-double knockdown primary astrocytes, accompanied by an increase in C26:0 levels. Recently it was also reported that increased leukotrienes and enhanced expression of 5-lipoxygenase (5-LOX) were detected in CCALD brain, which indicated that 5-LOX might have a role in the pathology of inflammatory demyelination in CCALD [102]. VLCFA accumulation directly increases 5-LOX expression and thereby mediates the production of leukotrienes, which might be involved in astrocytic proliferation and in the cell death of oligodendrocytes. Therefore, 5-LOX might be a new target for intervention in CCALD.

In oligodendrocytes, an excess of VLCFA might trigger ROS production or reduce the resistance against oxidative stress. It is possible that a dysfunction of ABCD1 has an adverse affect on mitochondria due to the accumulation of cellular acyl-CoA, which exacerbates oxidative stress in oligodendrocyte. By selectively inactivating *PEX5* in oligodendrocytes, Kassmann *et al.* showed that the peroxisomes in oligodendrocytes are essential for the preservation of axons and the maintenance of myelin [103]. Recently, Mastroeni *et al.* have reported that viral-based delivery of insulin-like growth factor-1 and neurotrophin-3, two potent inducers of myelination and oligodendrocyte survival, respectively, halt the progression of the disease [104]. Therefore, such a functional defect of oligodendrocytes would be a target for intervention in X-ALD patients. In CCALD, however, the process of demyelination might not be cell-autonomous, because astrocytes and microglia are known to be important for the support of axonal growth and myelination. In the CNS, the expression of ABCD1 occurs mostly in astrocytes and microglia in the subcortical and cerebellar white matter. Astrocytes play a major role against oxidative stress and protect oligodendrocytes and neurons. In addition, the remarkable efficacy of HSCT in halting cerebral demyelination suggests that a dysfunction of microglial ABCD1 could contribute to demyelination, because microglial cells are derived from myelo-monocytic hematopoietic cells. Eichler *et al.* have suggested that microglial apoptosis in perilesional white matter represents an early pathogenic change in CCALD [105]. Although the exact pathway for the production of ROS in X-ALD needs to be elucidated, chemical compounds that reverse the deregulated response to oxidative stress might provide a new therapeutic approach in X-ALD. Recently, it has been reported that valproic acid has a capacity to reduce the oxidative damage in peripheral mononuclear cells from X-ALD patients. Interestingly, valproic acid did not reduce saturated VLCFA, but did reduce monounsaturated VLCFA (C26:1), suggesting that mono-unsaturated rather than saturated VLCFA is involved in the production of ROS [75]. Therefore, this drug could be an attractive candidate for X-ALD.

FUTURE APPROACHES

At present allogeneic HSCT is the only effective treatment, when performed in the early stage of cerebral demyelination, although HSCT remains associated with a high mortality risk. Furthermore, recently Cartier *et al.* have reported that lentiviral-mediated gene therapy of hematopoietic stem cells (HSC) halted the progression of X-ALD in two X-ALD boys [106]. Cerebral demyelination was arrested 14 to 16 mouths after engraftment, and neurological and cognitive functions remained stable. Compared with allogeneic HSCT, the HSC-based gene therapy, if feasible, will provide more preferable treatment for CCALD. In addition to gene therapy, pharmacological therapies are of importance in the effort to delay of onset and the progression of the disease. Considering application of various type of X-ALD, reduction of cost and risk for the therapy, and protection effect against onset of the disease, development of pharmacological therapies has many advantages. As mentioned above, pharmacological up-regulation of *ABCD2*, stimulation of peroxisomal β-oxidation, suppression of VLCFA synthesis, and reduction of oxidative damage are all promising targets for future X-ALD therapy. As target proteins for X-ALD therapy have been highlighted by recent studies, effective compounds may be identified by means of high throughput screening. At the same time, as some candidate compounds have already been found, the synthesis and evaluation of the biological activities of their derivatives should be carried forward. However, the costs in time and money to develop a new drug are formidable. The screening of well-established drug substances thus seems to be a useful means to find candidate compounds for X-ALD, since it is expected that some of these compounds will exhibit some

previously unknown efficacy against the therapeutic targets of X-ALD.

Abnormal VLCFA accumulation seems to be necessary but not sufficient for the progression of the X-ALD pathologies. It has been speculated that in addition to the defect in the *ABCD1* gene, environmental factors and/or unidentified modifier genes modulate the disease severity, since the progression and clinical symptoms of the disease in brothers with the same mutation of the *ABCD1* gene were different. Therefore, one of the factors we must keep in mind are modifier genes. However to date, while several genes have been reported to be candidates, none have been identified as actual modifier genes. The identification of triggering and/or modifying factors is important for understanding both the pathogenic mechanisms and the targeting of therapeutic compounds.

Although the precise mechanism by which the degeneration of the CNS is effected in X-ALD patients remains to be elucidated, it seems likely that an attenuation of disrupted VLCFA metabolism and a decrease in oxidative damage in the CNS have the potential to both help delay the onset of disease and prevent the progression of neurological symptoms in X-ALD. At present, pharmacological induction of ABCD2 expression is a most reasonable and feasible therapeutic strategy for X-ALD. From a practical point of view, chemical compounds with the capacity to cross the blood-brain barrier and to induce *ABCD2* gene in brain, such as halogen-free thyromimetics and histone deacetylase inhibitors, is worthwhile for a realistic application for X-ALD patients.

FOOTNOTES

This research was supported in part by a Grant-in-Aid for the Research on Measures for Intractable Diseases from the Ministry of Health, Labour and Welfare of Japan, and for Scientific Research from the Ministry of Education, Culture, Sports, Science and Technology of Japan (18590049, 18790050, and 20590054). Pacific Edit reviewed the manuscript prior to submission.

ABBREVIATIONS

ALDP = Adrenoleukodystrophy protein

ABC = ATP-binding cassette

CNS = Central nervous system

DHEA = Dehydroepiandrosterone

DHA = Docosahexaenoic acid

ELVOL = Elongation of very long chain fatty acids

ER = Endoplasmic reticulum

HSCT = Hematopoietic stem cell transplantation

IBA = Indole-3-butyric acid

5-LOX = 5-Lipoxygenase

NBD = Nucleotide binding domain

4-PBA = 4-Phenylbutyrate

PPAR = Peroxisome proliferators-activated receptor

PMP70 = 70-kDa Peroxisomal membrane protein

PUFA = Polyunsaturated fatty acid

ROS = Reactive oxygen species

SREBP = Sterol regulatory element binding protein

TDM = Transmembrane domain

TR = Thyroid hormone receptor

VLCFA = Very long chain fatty acid

X-ALD = X-Linked adrenoleukodystrophy

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Received: May 16, 2010 Revised: August 17, 2010 Accepted: August 17, 2010

PMID: 21039332