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Review

Cerebral Inflammation in X-Linked Adrenoleukodystrophy

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Abstract. X-linked adrenoleukodystrophy (X-ALD) is an inherited neurodegenerative disease that affects approximately 1 in 25 000 males. It is characterized by elevated levels of saturated very long chain fatty acids (VLCFA), i.e., >C22:0, particularly in ganglioside and cholesterol ester fractions of brain white matter and adrenal cortex. Failure of peroxisomal very long chain fatty acyl-CoA synthetase (VLCS) to activate these VLCFA prevents their degradation by peroxisomal β-oxidation. X-ALD maps to Xq28[33] and the gene encodes a peroxisomal membrane protein[39, 40, 65] and not the gene for VLCS. The two most common forms of X-ALD are the cerebral (CER) form, with an inflammatory demyelinating reaction that resembles multiple sclerosis (MS), and adrenomyeloneuropathy (AMN), which involves the spinal cord and in which the inflammatory reaction is mild or absent. Investigations into the nature of the cerebral inflammatory demyelinating reaction in X-ALD will be the subject of this review.

Key words: X-linked adrenoleukodystrophy; inflammation; demyelination; cytokines; human leukocyte antigens.

Phenotypic Variability of X-ALD

The phenotypic presentation of X-ALD mutations is remarkably variable. Six phenotypes have been delineated in X-ALD hemizygotes that vary with respect to age of disease onset, the duration from age of onset to death, and the site of initial pathology.

The childhood cerebral form (CCER), in which there is a cerebral perivascular inflammatory demyelinating reaction, is the most common and severe form of X-ALD, occurring in 48% of approximately 1500 patients studied at the Kennedy Krieger Institute[38]. The mean age of onset of neurological symptoms in CCER is 7.1±1.7 years. In this illness, behavioral and cognitive deterioration progresses rapidly and patients typically die within 2 to 4 years of onset. In a small number of cerebral (CER) patients, cerebral involvement begins in adolescence (5%) or adulthood (3%) but is otherwise similar to CCER in respect to its clinical manifestations, rates of progression and the inflammatory response. AMN, with initial and primary involvement of the spinal cord and peripheral nerves, is the second most common form, occurring in 25% of patients in the Kennedy Krieger Institute series. AMN manifests with slowly progressive paraparesis and sphincter disturbances, involves long tracts in the spinal cord mainly, and appears to be a distal axonopathy with secondary demyelination. The inflammatory response is mild or absent. The mean age of onset in AMN is 27 years, but can occur as late as >60 years. Progression is slow and patients live well into adulthood. There is some cerebral involvement (as evidenced by magnetic resonance imaging data) in approximately 40% of AMN patients during the later stages of the disease[25].

Abbreviations used: X-ALD – X-linked adrenoleukodystrophy, AMN – adrenomyeloneuropathy, CCER – childhood cerebral form of X-linked adrenoleukodystrophy, CER – cerebral form of X-linked adrenoleukodystrophy.

The designation Addison-only (AD) is applied to patients with the biochemical defect of X-ALD who have primary adrenal insufficiency but are free of demonstrable neurological involvement. Some individuals with X-ALD mutations and elevated VLCFA have no clinical manifestations. Although late onset cannot be ruled out, asymptomatic individuals in their 60s have been observed.

In the other inherited lipidoses (e.g. metachromatic leukodystrophy, globoid cell leukodystrophy, Niemann-Pick disease, GM2 gangliosidosis) there is a correlation between the extent of biochemical abnormality and clinical phenotype. In X-ALD, however, no correlation between phenotype and VLCFA level (the biochemical abnormality) in plasma, fibroblasts or red blood cells, or fibroblast VLCFA β-oxidation has been reported in a large number of patients[1, 4, 36]. ANTOKU et al.[1] reported a difference in the mononuclear cell VLCFA ratio between 4 patients with childhood-adolescent CER and 4 patients with adult X-ALD (2 with AMN and 2 with adult CER).

It has been shown repeatedly that the same mutations in the X-ALD gene are associated with each of the six phenotypes[2, 21, 24, 39]. Thus, there is no correlation between genotype at the X-linked locus and phenotype. In addition, all phenotypes segregate in the same X-ALD kindreds and 50% of kindreds have both CCER and AMN[38]. This suggests that a factor other than the X-linked locus is involved in determining phenotype. Genetic segregation analysis of a large number of pedigrees and sib pair analysis independently suggest that an autosomal modifying gene determines whether or not individuals with X-ALD mutations develop cerebral inflammatory demyelinating lesions during childhood[37, 58].

The Cerebral Inflammatory Reaction in X-ALD

The pathogenetic mechanism of the cerebral inflammatory demyelinating reaction in X-ALD and the role of the protein product (ALDP) of the X-ALD gene at Xq28 in destructive demyelination are unknown. The inflammatory reaction in CER could be to an as yet unknown antigen (e.g. the accumulation of VLCFA or compounds containing VLCFA in the brain) with myelin-breakdown secondary to this inflammatory reaction. The mutated X-ALD protein might be antigenic but, as 69% of X-ALD patients (both CER and AMN) lack ALDP[9, 22, 65], it is clearly not required to initiate the cerebral inflammatory demyelinating reaction. Alternatively, elevated levels of VLCFA in myelin lipids in CER patients could lead to myelin instability and subsequent myelinolysis followed by an inflammatory reaction. Whatever the initiating event in the cerebral inflammatory demyelinating reaction in CER, it has been hypothesized that myelin breakdown products initiate a cytokine cascade which may lead to breakdown of the blood brain barrier followed by a cellular-based reaction to a central nervous system (CNS)-specific antigen (since there is no inflammatory reaction in the peripheral nervous system (PNS))[47].

The inflammatory nature of the cerebral demyelination seen in X-ALD sets it apart from the demyelination seen in the other inheritable leukodystrophies (e.g. metachromatic leukodystrophy, globoid cell leukodystrophy) and aligns it more with MS, the most common immune-mediated cerebral inflammatory demyelinating disease. POWERS et al.[48] showed that in CER inflammatory demyelinating lesions, reactive astrocytes, macrophages and T cells are the most prevalent cellular elements, with very few B cells present. They showed that in CER the inflammatory infiltrates are located behind the active lesion edge and, unexpectedly, in subsequent secondary tract degeneration. In contrast, the inflammatory infiltrates in MS are found at the active demyelinating lesion edge[53]. GRIFFIN et al.[12] identified both $CD4^+$ and $CD8^+$ T cells in CER lesions and also found a large number of B cells in perivascular cuffs. In CER, myelin lesions are usually bilaterally symmetrical and most severe in the occipital lobes, but they become more asymmetrical as they advance toward the frontal lobes[53]. Exceptions to this rule even within the same family are well documented[8].

Defects in CNS myelin are common in AMN. In contrast to CER, the lesions most commonly consist of foci of myelin pallor and loss with minimal reactive astrocytosis and lymphocytic infiltrates[48]. Sparse collections of macrophages, often striated and containing angulate lysosomes, are the most conspicuous cell reaction. SCHAUMBURG et al.[53] proposed that the major site of damage in AMN, rather than being the central or peripheral myelin, is more likely to be the axons of long spinal tracts. While some degree of myelinolysis and dysmyelination should occur concurrently with the axonal insult, in AMN this appears to usually remain a minor pathologic element[46]. However, in a minority of AMN patients, usually after many years of suffering from their myelopathy, some myelinolytic lesions progress to the inflammatory demyelination typical of CER.

In the destruction of the other cell types affected in X-ALD, i.e., adrenocortical cells, interstitial cells of Leydig, and Schwann cells, there are almost no inflammatory infiltrates. Free fatty acids are toxic to a variety of cells and their toxicity appears to increase with chain length. Thus, a cytotoxic pathogenesis has been pro-

posed for the adrenal, testicular and Schwann cell lesions in CER and AMN[48].

Cytokine Studies in X-ALD

The cerebral inflammatory reaction could be regulated at the level of inflammatory mediators (e.g. cytokines). Cytokines are antigen-nonspecific glycoproteins, synthesized and generally rapidly secreted in response to a stimulus. Cytokines are believed to act over a short range and to have very short half-lives. There are two distinct subtypes of $CD4^+$ T helper (Th) cells, Th1 and Th2, that are identifiable because each produces a different set of cytokines. Th1 associated cytokines are interleukin (IL)-2, IL-12, interferon (IFN)-γ and lymphotoxin (TNF-β) and Th2 associated cytokines are IL-4, IL-5, IL-6 and IL-10. Th1 cells are the major players in delayed-type hypersensitivity and in pro-inflammatory responses by activating cytotoxic T cells and macrophages, while Th2 cells induce B cell growth and differentiation and induce antibody production. The anti-inflammatory cytokines produced by the Th2 cells can down-regulate Th1 cell function.

We investigated the nature of the inflammatory demyelinating reaction in X-ALD by comparing cytokine gene expression in cerebral inflammatory demyelinative lesions in X-ALD and MS in an attempt to define the inflammatory demyelinating process in X-ALD. Expression of mRNAs of the cytokines TNF-α, IL-1β and IL-6 produced by macrophages (monokines), the lymphokines IFN-γ, IL-2, IL-4, IL-6 produced by T cells, and the TNF receptors [TNFRI (55 kDa) and TNFRII (75 kDa)] was measured in lesion samples taken *postmortem* from patients with CER and MS.

Our cytokine mRNA expression results for MS lesions confirmed immunocytochemical and *in situ* hybridization data in the literature showing that levels of the major inflammatory cytokines are elevated in MS brain lesions[6, 15, 16, 54, 67, 68]. We showed that expression of monokines and lymphokines is generally increased in MS lesions when compared with CER lesions or controls. IL-4, associated with Th2 cells, is involved in down-regulation of the cellular immune response. IFN-γ, associated with Th1 cells, enhances the cellular immune response. We showed that CER lesion samples do not have IL-4 mRNA, while MS patients have IL-4 mRNA in both acute and gliotic lesion samples. Also, MS patients have a decrease in IFN-γ in gliotic samples compared with acute samples, while in CER there is an increase in IFN-γ in gliotic lesion samples compared with CER acute lesion samples.

In our studies, the level of expression of a number of inflammatory cytokines in CER lesions is lower than that in MS lesions and more comparable with expression levels in control white matter samples. This suggests that transcriptional control of cytokine production in the inflammatory demyelinating reaction differs between CER and MS.

Tumor Necrosis Factor-α Studies in X-ALD

Several studies have implicated tumor necrosis factor-α (TNF-α), a cytokine, in the pathogenesis of a number of brain disorders, e.g. MS[15, 54], acquired immune deficiency syndrome (AIDS) encephalopathy[34, 66], bacterial meningitis[41], cerebral malaria[31] and X-ALD[29]. TNF-α is an important mediator of inflammation and has been shown to be capable of selectively damaging oligodendrocytes and myelin sheaths *in vitro*[54]. There is some controversy as to the significance of TNF-α in MS. Several groups have found elevated levels of TNF-α in cerebrospinal fluid and sera from MS patients[7, 13, 28, 56, 63]. Therapies aimed at down-regulating TNF-α appear promising for the treatment of MS[20]. However, other investigators found that the frequency of detectable cerebrospinal fluid and serum TNF-α was similar in MS patients and controls[10, 44, 45].

POWERS et al.[48] have shown that in acute inflammatory lesions from *postmortem* brain tissue of X-ALD patients with cerebral involvement, reactive astrocytes are most immunoreactive for TNF-α with macrophages staining less intensely. This is unusual, as in most pathologic conditions TNF-α production is associated primarily with macrophages. Astrocytes are most immunoreactive for TNF-α at the active demyelinating edge of the X-ALD lesion, with the inflammatory infiltrates (T cells and macrophages) located behind the active edge and in subsequent secondary tract degeneration. In MS, astrocytes are also most immunoreactive for TNF-α[15, 54] at the active demyelinating lesion edge, but in MS the inflammatory infiltrates are also located at the active lesion edge[48]. These data suggest that TNF-α immunoreactive astrocytes, at the lesion edge, may play a primary role in the inflammatory demyelinating process in CER. The prevalence of TNF-α positive astrocytes at the active demyelinating lesion edge in X-ALD, and even beyond in otherwise morphologically normal white matter, indicates some pathogenetic connection[48]. The observed decrease in astrocytic immunoreactivity in late acute to chronic active X-ALD lesions provides additional circumstantial evidence for a role in the early phase of demyelination.

In our laboratory we investigated TNF-α as a candidate modifying gene in X-ALD. We reported an in-

crease in TNF-α bioactivity in serum from patients with severe CCER. However, neither allelic differences in TNF-α nor levels of soluble TNF receptors accounted for the bioactivity differences or phenotypic heterogeneity in X-ALD[29].

In the earlier studies of POWERS et al.[48] it was unknown whether TNF-α was synthesized in astrocytes, accumulated in astrocytes or bound to astrocytes in CER lesions. Surprisingly, we showed that the level of TNF-α mRNA in CER acute/late acute lesion samples is only slightly above the level of detection[29]. However, using *in situ* hybridization, we demonstrated that TNF-α mRNA is localized within astrocytes and macrophages in CER patients[29]. Immunocytochemical localization of TNF-α in astrocytes has also been reported in MS by SELMAJ et al.[54] and in AIDS by TYOR et al.[64], although it is primarily localized to macrophages. Failure to detect TNF-α mRNA in total RNA from lesion cross sections together with the clear demonstration of TNF-α protein[48] and TNF-α mRNA[29] in astrocytes at the active demyelinating edge of inflammatory lesions implies that TNF-α expression is limited to a few reactive cells that might be important in the initiation of demyelination in the CER form of X-ALD. Failure to find allelic differences suggests that TNF-α does not account for the cerebral inflammatory response in X-ALD. Production of TNF-α by astrocytes may be followed by activation of macrophages and involvement of T cells in CER and progression of localized demyelination.

Human Leukocyte Antigen (HLA) Studies in X-ALD

Associations between HLA class I and class II alleles and various diseases have been reported by many investigators, e.g. in MS[11], systemic lupus erythematosus[14] and rheumatoid arthritis[42]. It is possible that in some X-ALD individuals an altered metabolite of ALDP (an endogenously synthesized antigen) might be presented by specific HLA class I molecules to $CD8^+$ cytotoxic T cells and thereby initiate an inflammatory reaction. Alternatively, if increased VLCFA in X-ALD brain tissue resulted in instability of myelin and subsequent myelinolysis, in some individuals, some of these myelin breakdown protein products (as exogenous antigens) might be presented by specific HLA class II molecules to $CD4^+$ T helper cells, thus initiating the inflammatory reaction. We studied HLA association within the major X-ALD phenotypes (CCER and AMN) by typing HLA class I and class II specificities on lymphoblastoid cells from X-ALD patients and controls to determine whether or not there is an association between HLA haplotype and X-ALD phenotype and compared our results to published findings in MS, the prototypical inflammatory demyelinating disorder[30]. However, when we compared HLA specificities in CCER and AMN we found no statistically significant associations with phenotype. Thus, HLA is not likely to be a primary determinant of phenotypic heterogeneity in this disease[30].

Biochemical Abnormalities in CER Brain

It has been proposed that VLCFA excess in brain tissue from patients with the cerebral form of X-ALD acts as the biochemical trigger for the inflammatory response, but the exact nature of this trigger has yet to be been defined. One of the difficulties in these studies has been the lack of availability of non-affected AMN brain tissue for comparison with non-affected CER brain tissue. Whereas a mild to moderate excess of VLCFA is present in all brain lipids in the CER form of X-ALD, the greatest excess occurs in the cholesterol ester, phosphatidylcholine (PC), ganglioside and proteolipid protein (PLP) fractions. Several authors have reported that there is an increased amount of cholesterol ester with an excess of VLCFA (C25:0, C26:0) in demyelinated regions of X-ALD brain[5, 17, 18, 23, 32, 35, 49, 50, 51, 61]. IGARASHI et al.[18], REINECKE et al.[52] and THEDA et al.[62] found a correlation between VLCFA level and degree of histologic abnormality in CCER brain and concluded that the cholesterol ester abnormality is a secondary phenomenon related to breakdown of myelin and subsequent release of free fatty acids, which become esterified with cholesterol and may serve a protective function. It is thus unlikely that cholesterol esterified with VLCFA acts as a trigger for the inflammatory response. Furthermore, OGINO and SUZUKI[43] showed that the degradation of cholesterol esters, including those that contain VLCFA, was unimpaired in X-ALD.

THEDA et al.[62] reported an excess in the C26:0 content of the PC fraction from apparently intact regions of brain tissue from one CCER patient. PC is a major constituent of white matter and myelin lipid in normal brain and is probably positioned on the external surface of the myelin sheath[62]. Location on the external surface of the myelin sheath would make a modified PC more accessible for recognition by the immune system. Neurologic disorders associated with anti-phospholipid antibodies have been described, but with clinical and neuropathological findings that differ from those in CCER.

VLCFA account for 28 to 50% of the fatty acids in X-ALD brain gangliosides in moderately affected white matter, compared with 2.5% in normal brain[17, 23]. C24:0

and C24:1 (and not C25:0 and C26:0) represent the predominant VLCFA in X-ALD gangliosides. LADISH et al.[26] showed that the length of the fatty acyl chain of the ceramide moiety dramatically influences ganglioside immunosuppressive activity. Gangliosides with shorter fatty acyl chains (C16:0), C18:0) are far more potent in inhibiting the *in vitro* lymphoproliferative response to a soluble antigen than are those containing longer fatty acyl chains (C22:0, C24:0, C24:1). Furthermore, unlike phospholipid components of membranes, gangliosides potentially can partition freely between membranes and the aqueous environment and thus passively migrate between cells. This type of ganglioside translocation could act as a physiological modulator of immune responses. Because some gangliosides, e.g. GM4, and PLP are unique to the CNS, i.e., not found in the PNS, and because the inflammatory response in the CER form of X-ALD is in the CNS only, gangliosides are potential triggers of the inflammatory response in the CER form of X-ALD.

In myelin, from both normally appearing and affected white matter from CCER brain, the proportion of saturated and mono-unsaturated VLCFA bound to PLP was increased at the expense of oleic acid (C18:0)[3]. PLP has recently emerged as a significant antigen in the prototypic demyelinating disease, MS, and as a potent encephalitogen in experimental models[59]. Inter-patient heterogeneity in PLP may contribute to phenotypic variation in X-ALD. One possible mechanism for this is alteration of the protein's antigenicity.

Conclusion

In summary, a number of candidate modifying genes in X-ALD (cytokines and HLAs) have been investigated, but none appear to account for the cerebral inflammatory demyelinating reaction and/or phenotypic heterogeneity observed. The pathogenetic mechanism of the cerebral inflammatory demyelinating reaction in X-ALD and the role of the protein product of the X-ALD gene at Xq28 in destructive demyelination are as yet unknown. The X-ALD protein, ALDP, is a peroxisomal membrane protein and a member of the ATP--binding cassette (ABC) transporter superfamily, ALDP is closely related to at least three other peroxisomal membrane proteins, PMP70[19], the X-ALD related protein (ALDR)[27] and the PMP70 related protein (P70R)[55]. ALDP forms homodimers and heterodimers with these other peroxisomal ABC proteins[57]. The genes for these three ALDP-related peroxisomal membrane ABC-proteins and for VLCS[60] are currently being investigated as potential modifier genes in X-ALD.

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