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Review

Acid ceramidase and human disease☆

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Abstract

Acid ceramidase (*N*-acylsphingosine deacylase, EC 3.5.1.23; AC) is the lipid hydrolase responsible for the degradation of ceramide into sphingosine and free fatty acids within lysosomes. The enzymatic activity was first identified over four decades ago, and is deficient in the inherited lipid storage disorder, Farber Lipogranulomatosis (Farber disease). Importantly, AC not only hydrolyzes ceramide into sphingosine, but also can synthesize ceramide from sphingosine and free fatty acids *in vitro* and *in situ*. This "reverse" enzymatic activity occurs at a distinct pH from the hydrolysis ("forward") reaction (6.0 vs. 4.5, respectively), suggesting that the enzyme may have diverse functions within cells dependent on its subcellular location and the local pH. Most information concerning the role of AC in human disease stems from work on Farber disease. This lipid storage disease is caused by mutations in the gene encoding AC, leading to a profound reduction in enzymatic activity. Recent studies have also shown that AC activity is aberrantly expressed in several human cancers, and that the enzyme may be a useful cancer drug target. For example, AC inhibitors have been used to slow the growth of cancer cells, alone or in combination with other established, anti-oncogenic treatments. Aberrant AC activity also has been described in Alzheimer's disease, and overexpression of AC may prevent insulin resistant (Type II) diabetes induced by free fatty acids. Current information concerning the biology of this enzyme and its role in human disease is reviewed within.

Keywords: Acid ceramidase; Farber disease; Ceramide; Apoptosis; Cancer

Contents

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1. Background and introduction

Acid ceramidase (*N*-acylsphingosine deacylase, EC 3.5.1.23; AC) was originally identified in rat brain homogenates by Gatt in 1963 [1], but the first substantial purification of the enzyme did not occur until 1995, when the protein was isolated from human urine [2]. The purified polypeptide was ~50 kDa and could be reduced into 13 and 40 kDa α- and β-subunits, respectively [2]. Peptido-*N*-glycanase and Endo-H further reduced the molecular weight of the β-subunit, suggesting the presence of 5 or 6 *N*-linked oligosaccharide chains. In contrast, the α-subunit was not glycosylated. These findings were subsequently confirmed by site-directed mutagenesis of the full-length AC cDNA [3].

Although the amount of purified, urinary enzyme obtained in these studies was small, it was enough to carry out preliminary characterization studies and to produce the first anti-AC polyclonal antibodies. These antibodies were subsequently used to investigate the enzyme's biosynthesis in cultured skin fibroblasts [3], revealing that AC was initially synthesized as a ~55 kDa precursor polypeptide that was proteolytically processed into the α- and β-subunits within lysosomes. A portion of newly synthesized AC also was secreted from the cells as a monomeric protein of ~47 kDa. At the present time the proteolytic enzyme(s) responsible for processing AC into the individual subunits remain unknown, as does the precise subcellular localization of the precursor and processed AC forms.

2. Isolation of AC cDNA and genomic sequences

The full length, human AC cDNA was isolated from cultured skin fibroblasts and pituitary tissue in 1996 [4]. The full-length sequence was 2312-bp and contained an open reading frame (ORF) of 1185-bp encoding a 395 amino acid polypeptide. Both the α and β-subunits were encoded by the same cDNA. The human AC gene, mapped to the short arm of chromosome 8 (p21.3–22), spanned ~30-kb and included 14 exons and 13 introns [5]. The murine AC cDNA was ~80% identical to the human cDNA, and the protein shared ~90% homology with its human counterpart [6].

3. Production and characterization of recombinant AC

To produce and characterize large quantifies of AC, the human enzyme was overexpressed in Chinese hamster ovary (CHO) cells by amplification of the transfected, full-length cDNA [7]. The majority of the overexpressed enzyme was secreted into the culture media and purified by sequential chromatography using Concanavalin A, Blue Sepharose and Superose 12. The purified, recombinant protein contained the same α- and β-subunits as human AC purified from natural sources (see above), had an acidic pH optimum when determined *in vitro* (4.5), and followed normal Michaelis–Menten kinetics using two different substrates, [^{14}C]-labeled C12 ceramide ([Lauryl-1-^{14}C]D-*erythro* sphingosine) and BODIPY-conjugated C12 ceramide. Deglycosylation studies showed that the purified, recombinant enzyme contained mostly "high mannose" type oligosaccharides which were present on the β-subunit. The β-subunit also had two distinct polypeptide backbones that differed in molecular mass by ~2–4 kDa. Amino acid sequence analysis revealed a single *N*-terminus, suggesting that the different peptide backbones were likely due to carboxy-terminal processing.

4. Identification and characterization of the reverse ceramidase activity

In 1963 Gatt suggested that partially purified, brain AC could synthesize ceramide from free fatty acids and sphingosine [1]. However, due to the substantial contaminants present in these early enzyme preparations, it remained unclear whether the hydrolysis ("forward") and "reverse" reactions were in fact carried out by the same polypeptide. In 2003 Okino et al. [8] showed that the highly purified, recombinant AC could carry out the reverse reaction *in vitro* using [^{14}C]-labeled C12 fatty acid ([1-^{14}C]lauric acid) and sphingosine as substrates. In contrast to the degradative reaction, the "reverse" activity occurred at an optimal, *in vitro* pH of ~6.0. Non-ionic detergents and zinc cations inhibited this activity, whereas most other cations were stimulatory. Of note, sphingomyelin also was very inhibitory towards this reaction, while the anionic lipids, phosphatidic acid and phosphatidylserine, were stimulatory. Of various sphingosine isomers tested in the reverse reaction, only the natural, D-*erythro* form could efficiently serve as a substrate in the reverse reaction. The preferred fatty acid was lauric acid.

To confirm these findings, reverse ceramidase activity was determined in cell lysates from a Farber disease patient, and was reduced to the same extent as the degradative activity [8]. This demonstrated that both the forward and reverse AC activities were derived from the same gene product. Furthermore, when fluorescently (NBD)-conjugated lauric acid and sphingosine were added to cultured lymphoblasts from a Farber disease patient, the conversion to NBD-ceramide was reduced ~30% when compared to normal cells. Thus, these results confirmed initial observations reported over 40 years ago on the reverse ceramidase reaction, and further revealed that AC plays a critical role in the regulation of ceramide and sphingosine metabolism.

5. Characterization of a multienzyme complex containing AC, acid sphingomyelinase and β-galactosidase

Notably, when media from CHO cells genetically engineered to over-express human AC were analyzed, a large amount of acid sphingomyelinase (ASM) activity was found [7]. Compared to parental CHO cells, the increase in ASM activity was 20-fold or greater. This was not due to increased expression of the CHO ASM gene, and it was therefore reasoned that some of the endogenous, CHO-derived ASM was associating with the overexpressed, human AC in these cells, and that the two enzymes were being released into the culture media together. To confirm that this was not an artifact of the

CHO cell overexpression system, human AC was similarly overexpressed in Farber disease cells, and the same observations were made.

Anti-AC antibodies were subsequently used to immunoprecipitate AC from the CHO and normal skin fibroblast cell culture media, and the activities of several other lysosomal hydrolases were measured. In addition to AC activity, high levels of ASM activity were found in these precipitates, as well as the activity of β-galactosidase [7]. These findings were important since they revealed: (a) that the association of AC and ASM was strong enough to permit co-precipitation, and (b) at least one other enzyme was present in this complex (β-galactosidase). It is notable that there are two β-galactosidases in cells, one of which uses galactosylceramide as substrate. It is also important to note that the activities of several other lysosomal enzymes (e.g., alpha iduronidase, alpha galactosidase, etc.) were not elevated in these immunoprecipitates, indicating the specificity of this complex.

6. Farber disease

The first case of Farber disease (Farber Lipogranulomatosis; FD) was described and characterized in 1957 by Sidney Farber [9], but it was not until 1972 that the inherited deficiency of AC activity was found to be the cause of this disorder [10] (Table 1). Based on the clinical severity and sites of major tissue involvement, FD has been classified into 7 subtypes [11]. In most cases, the disease is diagnosed early in life with typical symptoms including deformed joints, subcutaneous nodules, and progressive hoarseness. Type 1 patients have a very severe phenotype and most die by 2 years of age. A majority have neurological involvement in addition to the characteristic findings mentioned above. Types 2 (Intermediate) and 3 (Mild) FD patients have less severe phenotypes and may survive longer. Type 4 FD is an extremely rare, neonatal form of the disease that presents in infancy with hepatosplenomegaly and leads to death by 1 year of age. Only 6 patients with this form have been described. Type 5 FD is characterized by progressive neurologic deterioration and survival up to 5 years of age. Type 6 FD is an unusual entity in that only one patient has been described who inherited mutations in two distinct genes, acid ceramidase and the β-subunit of hexosaminidase. Type 7 FD is actually not FD at all, but rather occurred in a patient carrying mutations in the gene encoding the prosaposin polypeptide. Since there was a profound reduction in AC activity in this individual and clinical symptoms similar to FD, this "experiment of nature" demonstrated for the first time that AC requires an intact prosaposin gene for *in vivo* function [12,13]. Subsequent studies went on to demonstrate that SAP-D was required for optimal AC activity [14,15].

FD is diagnosed by the demonstration of reduced AC activity, abnormally high ceramide levels in cultured cells, biopsy samples or urine, and the presence of "Farber bodies", comma-shaped curvilinear tubular structures, by electron microscope analysis. FD is a very rare autosomal recessive disorder, and less than 100 cases have been reported since the first description in 1957. Due to the extremely small patient population, the natural history and underlying pathophysiology of this disease is poorly described.

Table 1
Historical landmarks in the study of acid ceramidase

1963	Enzyme first discovered and partially purified by Gatt
1963	"Reverse" ceramidase reaction first identified
1972	Deficiency of acid ceramidase first found in Farber disease patients
1995	First substantial purification of acid ceramidase from human urine
1996	First cloning of the human acid ceramidase cDNA
1996	First mutation identified in a Farber disease patient
1999	First cloning of the human acid ceramidase gene
2002	First production of recombinant, human acid ceramidase
2002	First acid ceramidase "knock-out" mouse model constructed

To date a total of 17 different mutations in the AC gene have been found in FD patients ([4,5,16–18]; see Fig. 1). Of these 17 mutations, 12 are point mutations leading to single amino acid substitutions, 3 are due to small deletions within the AC gene, 1 is due to a premature termination, and 1 results in an abnormal fusion protein. As with other storage disorders, the distinct clinical presentation of individual FD patients is likely due to the specific mutations inherited in the AC gene. However, at the present time the clinical course cannot be accurately predicted from the genotype, nor can it be closely correlated with the amount of residual AC activity in cultured cells.

7. Construction and characterization of an AC "knock-out" mouse

A complete "knock-out" AC mouse model was created in 2002 by Li et al. [19]. To generate this model, an AC targeting vector was constructed and used to obtain F1, heterozygous animals. Notably, genotype analysis of over 150 offspring or embryos from these heterozygous intercrosses revealed an absence of −/− individuals at developmental day E8.5 or later. Thus, the first important observation from these studies was that homozygosity for the mutant allele led to an early, embryonic lethal phenotype. This observation was confirmed by generating a second line of AC knock-out mice using a different, targeted ES cell clone. There were two possible explanations for this outcome: either the −/− embryos were not being formed (due to changes in the −/− gametes that prohibited fertilization), or the embryos were being formed, but died before E8.5. Of note, several resorbed embryos were observed in the pregnant females from these matings, but these could not be accurately genotyped due to maternal contamination following dissection. Studies are ongoing to investigate the mechanism(s) leading to the lethal phenotype.

The second surprising observation obtained from these studies was that as the +/− breeding pairs were mated over time, the ratio of +/+ to +/− pups from these intercrosses shifted from the expected 1:2 ratio to a ratio of 1:10 or more. Thus, as the +/− animals aged, they generated predominately +/− offspring. This observation also was confirmed by analysis of the second, independent AC knock-out line. One hypothesis to explain this finding is that as +/− animals age, they accumulate lipids in their

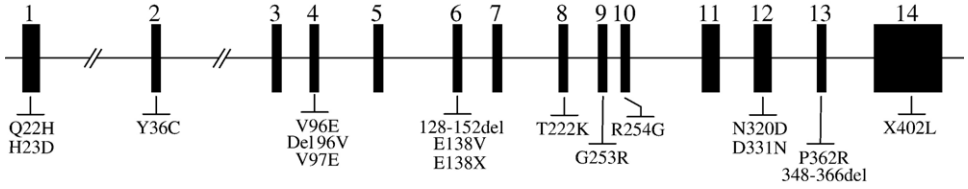

Fig. 1. Schematic depiction of the known mutations in the human acid ceramidase gene causing Farber disease. The 14 exons of the gene are indicated by the black boxes. Mutations are listed below the exons.

mutant gametes that enhance the likelihood of fertilization. This hypothesis is supported by the observation that several organs of +/− animals, including ovarian tissue, exhibited substantial lipid storage over time [19]. One working theory is that the mutant gametes (male or female) in +/− parents may have abnormal lipid membranes (presumably more hydrophobic due to sphingolipid and cholesterol storage), and that this, in turn, may provide them with a fertilization advantage.

Of particular relevance to these findings is the fact that ceramide metabolism plays a key role in oocyte development [20]. For example, in the human ovary ceramide levels are increased immediately following menopause, when a woman's pool of oocytes has been almost entirely exhausted. Since the process of oocyte loss occurs from apoptosis, it is presumed that ceramide accumulation is responsible, at least in part, for this observation. Moreover, work using acid sphingomyelinase "knock-out" mice has shown that oocytes lacking this enzyme are resistant to stimuli promoting apopotosis, presumably due to a dysfunctional ceramide response [20]. Sperm from these same mice also have lipid abnormalities, leading to profound morphological and functional defects [21]. Thus, these and other studies suggest that ceramide metabolism plays a key role in gamete formation and function, perhaps explaining, at least in part, observations described above on the AC knock-out mice.

8. Acid ceramidase and cancer

Based on the important role(s) of ceramide and sphingosine in regulating cell growth, these sphingolipids have become important targets in cancer therapy (for review see [22]). Moreover, since AC is one of the key enzymes regulating sphingolipid metabolism, several of these recent therapies have been focused on this enzyme. For example, overexpression of AC in murine L929 fibrosarcorma cells suppressed apoptotic cell death induced by tumor necrosis factor alpha (TNFα) [23], presumably by reducing ceramide levels. Moreover, a recombinant Sindbis virus (SV) expressing AC decreased intracellular ceramide and protected cells from SV-induced apoptosis [24].

Conversely, inhibitors of AC activity may lead to increased ceramide levels and stimulate apoptotic cell death. For example, *N*-oleoylethanolamine, an inhibitor of AC activity, increased ceramide formation and enhanced apoptosis in L929 cells [23], and also enhanced tumor-induced apoptosis in dendritic cells (DC) [25] and radiation-induced apoptosis in human glioma cells [26]. In addition, *N*-oleoylethanolamine treatment led to increased ceramide levels and TNF-α/IFN-α-induced apoptosis in primary placental trophoblasts [27].

Another inhibitor of AC, B13 (1R,2R or 1S,2S-(2-N-myristoylamino-1-(4-nitrophenyl)-1,3-dihydroxypropan), has been shown to induce apoptosis in prostate cancer cells [28]. Of particular importance, the growth of prostate cancer xenografts was efficiently inhibited compared to conventional radiation therapy when mice were treated with the combination of B13 and radiation, defining a synergistic role for these inhibitors with established cancer treatments. Treatment of nude mice with B13 alone also prevented tumor growth using two different aggressive human colon cancer cell lines metastatic to the liver, without any toxicity to normal cells [29].

Other ceramide analogs, (1S,2R)-2-N-myristoylamino-1-phenyl-1-propanol (De-MAPP) and (1R,2R)-2-N-myristoylamino-1-(4-nitrophenyl)-1,3-propandiol (D-NMAPPD), also suppressed AC activity and led to the elevation of endogenous ceramide levels. Subsequently, apoptosis was increased in primary keratinocytes [30]. De-MAPP also enhanced nerve growth factor-induced apoptotic cell death in hippocampal neurons [31].

These findings and others suggest that the inhibition of AC activity may serve as a useful target for cancer therapy, alone or in combination with other anti-oncogenic treatments. However, it is important to recognize that the mechanism(s) by which these inhibitors are functioning, as well as their specificity towards the individual ceramidases, remains unclear. For example, De-MAPP has been reported to inhibit AC activity, but also inhibits the activities of alkaline and/or neutral ceramidases in some cell lines. Furthermore, *N*-oleoylethanolamine and B13 appear to have preferential inhibition towards AC activity, but in some reports inhibition of other ceramidases has been shown. Therefore, although the biological effects of inhibiting ceramidase activities are well documented, it cannot always be concluded from these studies which ceramidase(s) is responsible for the effect. Also, in most cases the inhibitory compounds have not been tested with pure enzymes, and thus the mechanism of inhibition remains unknown. Lastly, and quite importantly, lysosomal ceramide does not appear to play an essential role in stress-induced apoptosis [32,33], or the apoptosis induced by exogenous ceramide treatment [34]. Whether or not AC activity contributes to levels of non-lysosomal ceramide also remains unknown, although it should be noted that acid sphingomyelinase, another "lysosomal" enzyme that closely associates with AC, certainly does (e.g., [35,36]). To better understand the role of AC in apoptosis, a more complete understanding of its trafficking and subcellular localization is certainly required, particularly in tumor cells and

normal cells subjected to stress. In addition, better characterization of the reagents used to inhibit AC activity is needed.

Also of interest, abnormal expression of endogenous AC has been reported in several human cancers. For example, overexpression of AC was reported in prostate cancers [37] and melanomas [38]. In addition, high levels of AC mRNA, protein, and activity were found in human alveolar macrophages that have an extended lifespan compared to precursor monocytes. Moreover, inhibition of AC activity induced cell death earlier than expected in these macrophages [39]. These results also suggest an important role of AC in tumorigenesis and/or in prolonging the survival of human cells.

Thus, overall there appears to be a correlation between cell growth and AC activity; i.e., high AC activity leads to reduced ceramide levels and enhanced cell growth, while low AC activity leads to reduced cell growth through an enhanced ceramide response. However, it must be noted that there are exceptions to this "simple" rule, such as decreased or shut-down AC expression in thyroid [40] and gastrointestinal cancers [17]. Clearly, much more information is needed concerning the function of AC in live cells, particularly with regard to the enzyme's processing and subcellular trafficking, and how its location in different cellular compartments influences downstream ceramide and sphingosine signaling events.

9. Acid ceramidase and other complex diseases

Aside from cancer, AC may play a role in the pathology of other, important complex diseases. For example, recent studies have shown that insulin resistance and type 2 diabetes result from the accumulation of lipids in tissues not normally associated with fat storage, and that free fatty acids inhibit insulin stimulation of Akt/protein kinase B, a central regulator of glucose uptake and anabolic metabolism through stimulation of ceramide production [41]. Importantly, it also has been shown that AC overexpression prevents the inhibitory effects of saturated fatty acids on insulin signaling [42], presumably through the reduction of ceramide. Of particular note, in this study overexpression of AC not only reduced ceramide levels, but also promoted accumulation of sphingosine.

In another recent publication it has been suggested that AC might play a role in the etiology of Alzheimer's disease [43]. In this study the major glycoproteins from Alzheimer's disease brains were obtained, and among these AC activity and protein levels were significantly elevated. Immunohistochemical staining indicated that accumulating AC was located mainly in the cell bodies of neurons and co-localized with neurofibrillary tangles. These intriguing findings require further investigation, but also suggest that AC-mediated signaling pathways might be involved in the molecular mechanisms leading to Alzheimer's disease.

10. Conclusions

AC plays a central role in sphingolipid metabolism, particularly in controlling the levels of ceramide and sphingosine in cells. Since its original description over 40 years ago, numerous advances have been made. A human disease (FD) has been associated with AC deficiency, and the first "knock-out" mouse model of AC deficiency has been produced. The gene encoding AC has been obtained, numerous mutations in this gene causing FD have been identified, and large quantities of recombinant enzyme have been produced and characterized. Several anti-AC antibodies are also available and have been used to begin investigating the cell biology of this important protein. However, despite these important advances, much more information is required to fully understand the role of AC in sphingolipid-mediated signal transduction and human disease. Important questions that will be addressed during the upcoming years include: (a) how AC is trafficked in cells and the influence of its subcellular location on the "forward" and "reverse" AC activities, (b) how AC function is affected by its presence in a multienzyme complex, (c) how AC influences fertilization and early mammalian development, and (d) what role AC plays in the etiology of complex human diseases. Moreover, based on our continuing understanding of AC's biology and its important role in the regulation of sphingolipid metabolism, this enzyme is likely to continue to be a target for new drug development in cancer and other disorders.

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