Ceramide in *Pseudomonas aeruginosa* Infections and Cystic Fibrosis

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Key Words

Sphingomyelinase • Ceramide • Cystic fibrosis • *P. aeruginosa*

Abstract

Cystic fibrosis is one of the most common autosomal recessive disorders, at least in Western countries. Patients with cystic fibrosis exhibit many symptoms, the most important of which are chronic inflammation and bacterial infections of the lung. Cystic fibrosis is caused by mutations of the cystic fibrosis conductance regulator (*CFTR*) gene; however, the molecular mechanisms leading to the clinical manifestations of cystic fibrosis are still unclear. Here we discuss recent findings related to the role of sphingolipids, in particular ceramide, in cystic fibrosis and the bacterial infections associated with that disease. Ceramide accumulates in the lungs of cystic fibrosis mice and causes pulmonary inflammation, infection, and cell death, events that are corrected by the genetic deletion or pharmacological inhibition of acid sphingomyelinase; this inhibition normalizes ceramide concentrations in murine models of cystic fibrosis. Initial clinical studies suggest that pharmacological inhibition of acid sphingomyelinase

may be a novel strategy for treating patients with cystic fibrosis.

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Cystic fibrosis

Cystic fibrosis is caused by mutations of the cystic fibrosis transmembrane conductance regulator (*CFTR*) gene [1-3]. The gene frequency is approximately 1/50 (0.02); therefore, the disease affects 1 person for approximately every 2500 births. Altogether, approximately 40,000 children and young adults in the European Union and a similar number in the United States are affected. Thus, cystic fibrosis is the most common autosomal recessive disorder in these regions.

Many mutations in the *CFTR* gene have been found to induce cystic fibrosis; however, the mutations that cause most clinical cases are those of amino acids 508 and 551 [1-3]. Genetic defects of the *CFTR* molecule cause many clinical symptoms, the most important of which are pulmonary and gastrointestinal symptoms. Gastrointestinal symptoms include malabsorption, intestinal obstruction, defects in the secretion of pancreatic enzymes, pancreatitis, and alterations in the liver. At present, the

pulmonary problems are most important for the disease course and for patients' life expectancy. Almost all patients experience recurrent and chronic infections with *Pseudomonas aeruginosa* and *Staphylococcus aureus*, but infections with *Burkholderia cepacia* and *Haemophilus influenzae* are also common [4]. The results of a recent survey from the US cystic fibrosis registry show that most patients are first infected with *S. aureus* but that *P. aeruginosa* infections are very common among older patients [4].

The details of the pathophysiological mechanisms that mediate the high sensitivity of cystic fibrosis patients to pulmonary infections are unknown. Because CFTR exhibits chloride channel activity, researchers speculated that the sensitivity to infection is related to changes in water absorption by the mucus found on epithelial cells of the respiratory tract [5]. Changes in the mucus would then result in a reduction in mucociliary clearance. However, many studies have demonstrated that the disease is much more complex and includes impairments in the mucociliary clearance system and cationic antimicrobial (poly) peptides, i.e. defensins, alterations in the function of neutrophils and macrophages, alterations in the mucus that lines the respiratory tract, changes in bacterial internalization into epithelial cells lacking CFTR, alterations in the frequency of cell death induced in mammalian cells by pathogens, and changes in lipid metabolism [5-11]. In the present review we will focus on the role of sphingolipids in *P. aeruginosa* infections and cystic fibrosis.

Membrane rafts

In 1972, Singer and Nicolson described the fluid mosaic model of the cell membrane; this model suggested that the cell membrane is in a liquid-disordered phase [12] and that proteins are randomly distributed in the cell membrane. However, biophysical studies have demonstrated that the cell membrane contains very small, distinct domains that are generated by the preferential interaction of sphingolipids with each other and with cholesterol and that these domains exist in a liquid-ordered phase [13-15]. The interactions between the headgroups of (glyco)sphingolipids and the hydroxy group of cholesterol are hydrophilic; in contrast, the binding between acyl-chains of sphingolipids and the sterol ring system involves hydrophobic van der Waal interactions. These binding forces result in a relatively tight interaction between these lipids and in the spontaneous formation of

domains that are in the liquid-ordered phase or even of gel-like domains with higher melting temperatures than other phospholipids in the cell membrane [13-15]. Cholesterol molecules not only interact with sphingolipids but also seem to fill the void spaces between bulky sphingolipids and, sterically, to stabilize sphingolipid- and cholesterol-enriched domains [16, 17]. These distinct sphingolipid- and cholesterol-enriched membrane domains are called rafts [13]. Recent microscopy studies of cell membranes using the STET technique suggest that these rafts have a diameter of less than 20 nm [18]. Rafts are very likely modified during cell activation or infections, respectively, and some of the current concepts about the mechanisms how rafts are modified during cell activation are discussed in the next paragraph.

Ceramide-enriched membrane domains and platforms

The most prevalent membrane sphingolipid in rafts is sphingomyelin, which consists of a very hydrophobic ceramide moiety (a D-erythro-sphingosine bound to a fatty acid by an amide ester) and a hydrophilic phosphorylcholine headgroup [19]. The acyl chain of the fatty acid may contain 2 to 32 carbon atoms, but the acyl chains of physiological ceramides contain 16 to 32 carbon atoms. The hydrolysis of sphingomyelin, which is catalyzed by the activity of acid, neutral, or alkaline sphingomyelinase, results in the generation of ceramide [20-22]. Ceramide is generated not only by sphingomyelinases [for review see 15] but also by a de novo synthase pathway [23], by the retrograde activity of the enzyme ceramidase that mediates the synthesis of ceramide from sphingosine [24], by the hydrolysis of complex glycosylated lipids [25], and by the hydrolysis of ceramide 1-phosphate (Fig. 1) [26]. The de novo synthesis, which begins with the condensation of serine with palmitoyl-CoA, is catalyzed in the cytoplasmic leaflet of the endoplasmic reticulum. The hydrophobic ceramide binds to ceramide transfer protein (CERT), which transports ceramide to the Golgi apparatus [27].

Acid sphingomyelinase hydrolyzes sphingomyelin to ceramide, preferentially at an acidic pH. However, because other lipids crucially influence the Michaelis-Menten constant (K_m) of the enzyme to its substrate, the enzyme seems to be able to hydrolyze sphingomyelin under almost neutral conditions [28, 29]. Because acid sphingomyelinase resides in lysosomes and secretory lysosomes in mammalian cells [28-32], the cellular

Fig. 1. Pathways leading to the formation of ceramide. Ceramide is formed by hydrolysis of sphingomyelin, degradation of glycosylated sphingolipids, by the de novo pathway or by retrograde activity of the acid ceramidase. Important enzymes are indicated in *italic*.

distribution of the enzyme is complex. Most of the enzyme seems to reside in classic lysosomes, where it mediates the catabolism of sphingomyelin.

A genetic defect in acid sphingomyelinase results in the accumulation of sphingomyelin and the occurrence of a lysosomal storage disorder called Niemann-Pick disease type A or B. However, the pool of acid sphingomyelinase in secretory lysosomes seems to participate in signal transduction events [28-32]. Mobilization of these vesicles triggers the fusion of secretory lysosomes and the cell membrane, and this fusion results in the exposure of acid sphingomyelinase on the outer leaflet of the cell membrane [31, 32]. Activation of several receptors, such as CD95, DR5, CD40, and the platelet-activating factor (PAF) receptor, but also some bacterial and viral infections or stress stimuli, trigger the surface exposure of acid sphingomyelinase [30, 33-41]. Finally, a change in the glycosylation pattern of acid sphingomyelinase results in the expression of a secretory acid sphingomyelinase that is secreted upon stimulation, for instance by activation of interleukin (IL)-1 receptors [28, 29].

Surface acid sphingomyelinase and secretory acid sphingomyelinase generate ceramide molecules on the outer leaflet of the cell membrane. Ceramide molecules dramatically alter the biophysical properties of the membrane, because these molecules spontaneously associate with each other to form small, highly hydrophobic, and ordered ceramide-enriched membrane domains [15, 17]. These small domains spontaneously fuse to larger ceramide-enriched membrane domains, which are called ceramide-enriched membrane platforms; these platforms may reach a diameter of several

micrometers. Ceramide-enriched membrane domains may aid the reorganization of receptors and signaling molecules; however, ceramide may also be involved in direct activation of cellular enzymes. Because the composition and fluidity of ceramide-enriched membrane domains differ from those of other parts of the cell and those of the intracellular membranes, ceramide-enriched membrane domains may permit the trapping and aggregation of receptors and signaling molecules within these membrane domains. We have previously demonstrated that receptors such as CD95, DR5, and CD40 [30, 34, 35] cluster in ceramide-enriched membrane domains upon activation. At present, the mechanisms that mediate the sorting of activated receptors into ceramide-enriched membrane domains are unknown, although some findings suggest that the length and the charge of the transmembrane domain determine the trapping of receptors in ceramide-enriched membrane platforms [42]. The reorganization of a receptor within ceramide-enriched membrane domains may also result in a preferential interaction with downstream signaling molecules, a concept that has been demonstrated for CD95 and caspase 8 [42]. In addition, ceramide-enriched membrane domains may mediate the recruitment, clustering and activation, of a certain set of signaling molecules mediating the specific effect of the cognate receptor. This was demonstrated for instance for nicotinamide adenine dinucleotide phosphate (NADPH) oxidase [43], which clusters and becomes activated after infection of cells with *P. aeruginosa*. However, future studies will be needed to define how ceramide-enriched membrane platforms initiate a specific response.

Ceramide also directly regulates the activity of signaling molecules, in particular cathepsin D, phospholipase A₂, kinase suppressor of Ras (KSR; identical to ceramide-activated protein kinase), serine/threonine ceramide-activated protein phosphatase (CAPP), protein kinase C isoforms, and c-Raf-1 [44-49]. Furthermore, ceramide has been shown to inhibit the potassium channel Kv1.3 and the calcium release-activated calcium (CRAC) channel in lymphocytes [50-53]. Finally, ceramide-molecules seem to form channels or pores in the outer mitochondrial membrane [54], and these structures may be involved in apoptosis.

Bacterial infections and ceramide

An association between ceramide, the acid sphingomyelinase and bacterial infections was first

demonstrated for *Neisseriae gonorrhoeae* [38, 55]. These studies demonstrated that acid sphingomyelinase is activated and ceramide is released when human cells are infected with *N. gonorrhoeae*. Studies using acid sphingomyelinase-deficient cells obtained from patients with Niemann Pick disease Type A demonstrated that acid sphingomyelinase and ceramide are required for the internalization of *N. gonorrhoeae* [38]. These paradigmatic experiments were extended to studies of the role of sphingolipids in infection with *Escherichia coli*, *S. aureus*, *Listeria monocytogenes*, *Salmonella typhimurium*, and *Mycobacterium avium* [56–60]. These studies demonstrated the activation of acid sphingomyelinase and the concomitant release of ceramide upon infection of endothelial cells with *S. aureus*, the infection of human embryonal kidney cells with *E. coli*, the infection of macrophages with *L. monocytogenes* or *S. typhimurium*, and the infection of mononuclear cells with *M. avium* [56–60]. In addition to mediating the internalization of pathogens, ceramide has been shown to be also crucial for the induction of cell death, at least upon *S. aureus* infection [57]. Elegant studies have demonstrated that P-fimbriae of *E. coli* bind to glycosphingolipids and that this binding induces the formation of ceramide [61]. Ceramide in turn mediates stimulation of Toll-like receptor 4 to trigger the effects of the infection [61, 62].

P. aeruginosa infections play an important role in the morbidity and mortality of patients with cystic fibrosis, but they are also very important in pulmonary infections of immunocompromised patients, wound infections, and sepsis [63]. Once patients with cystic fibrosis have passed early childhood, a very high percentage of them experience recurrent and, finally, chronic *P. aeruginosa* pneumonia [4]. More than 80% of adult patients with cystic fibrosis are infected with this pathogen, a finding illustrating the high clinical relevance of *P. aeruginosa*.

Our results demonstrate that the infection of murine or human respiratory epithelial cells and macrophages with *P. aeruginosa* triggers the activation of acid sphingomyelinase and the release of ceramide within a few minutes after infection [37]. However, the time course of ceramide release upon infection depends on the strain of *P. aeruginosa*: the release of ceramide is much faster after infection with very virulent strains and slower after infection with less pathogenic strains [37, 64]. The release of ceramide upon *P. aeruginosa* infection results in the formation of large ceramide-enriched membrane platforms that co-localize with *P. aeruginosa* during infection of the cell [37]. Studies using cells

genetically deficient in acid sphingomyelinase have demonstrated that acid sphingomyelinase is required for the formation of ceramide-enriched membrane platforms, internalization of the bacteria, and induction of cell death. None of these events occur in cells lacking acid sphingomyelinase upon *P. aeruginosa* infection, although these events are readily detectable in wild-type cells. Similar results have been obtained after pulmonary infection of acid sphingomyelinase-deficient and wild-type mice: Although *P. aeruginosa* is internalized into epithelial cells and triggers the death of bronchial epithelial cells in wild-type mice, these events are not induced in acid sphingomyelinase-deficient mice [37]. However, the release of cytokines, which is moderately increased in the lungs of wild-type mice upon infection, is uncontrolled and exaggerated in acid sphingomyelinase-deficient mice infected with *P. aeruginosa*. The uncontrolled release of cytokines in infected acid sphingomyelinase-deficient mice finally results in a cytokine storm and the death of the mice. These findings indicate that ceramide-enriched membrane platforms are crucially involved in the infection of mammalian epithelial cells with *P. aeruginosa* and the control of cytokine release [37]. At present, the mechanisms that mediate the activation of acid sphingomyelinase by *P. aeruginosa* require definition.

Ceramide-enriched membrane domains cluster CD95 receptor and cystic fibrosis transmembrane conductance regulator (Cftr) molecules upon cellular infection with *P. aeruginosa* [37]. Likewise, a partitioning of Cftr into rafts has been observed in cells infected with *P. aeruginosa* [65]. Clustering of CD95 in ceramide-enriched membrane platforms may be involved in *P. aeruginosa*-induced cell death, although this concept has not yet been proved. CD95 has been demonstrated to be crucially involved in the induction of cellular apoptosis and bronchial epithelial cell death in vivo upon infection with *P. aeruginosa*. A defect in apoptosis in CD95-deficient animals results in a very high susceptibility of these animals to *P. aeruginosa* infections [66]; this finding suggests that the induction of apoptosis in epithelial cells is central to a coordinated defense against pulmonary infections with *P. aeruginosa*. At present, it is unknown how apoptosis regulates the local defense to the pathogen, but it has been speculated that apoptotic cells down-modulate the inflammation and prevent an overshooting response. It is very interesting to note that recent studies by Kumar and colleagues with two independent cohorts of cystic fibrosis patients have demonstrated that allelic variants within intron 2 of the CD95 gene modulate the manifestation of cystic fibrosis [67]. This finding suggested

Fig. 2. *Cftr*-deficiency results in ceramide accumulation in bronchial epithelial cells. Paraffin sections of lungs from wildtype and *Cftr*-deficient mice were stained with Cy3-coupled anti-ceramide antibodies. The samples were analysed by confocal microscopy. The data show a marked accumulation of ceramide in epithelial cells of *Cftr*-deficient bronchial epithelial cells compared to those in wildtype mice.

that CD95-mediated signalling or apoptosis plays a role in *P. aeruginosa* infections in cystic fibrosis patients. The clustering of CFTR might be involved in internalization of *P. aeruginosa* [9, 37, 68], since CFTR functions as a receptor for *P. aeruginosa* [68]. Lipopolysaccharide molecules of *P. aeruginosa* have been shown to bind to a short amino acid sequence in CFTR (AA 103-117), and a deficiency in CFTR or the blockade of this binding motif prevents the internalization of *P. aeruginosa* [68].

In summary, ceramide-enriched membrane domains that are formed upon the infection of mammalian cells with *P. aeruginosa* may serve to cluster cellular receptors such as CD95 and *Cftr* and, thus, to trigger cell death and internalization of the pathogen. However, it is very likely that ceramide-enriched membrane domains play additional, currently undefined roles in the infection of epithelial cells with *P. aeruginosa*. In particular, the mechanisms by which ceramide-enriched membrane platforms control the release of cytokines in the lungs of acid sphingomyelinase-deficient mice infected with *P. aeruginosa* require definition.

The results of studies using fluorescence-labeled cholera toxin suggest that, upon infection, ceramide-enriched membrane platforms are generated from the small membrane rafts described above [37]. Infection of epithelial cells triggers translocation of CFTR into the raft fraction [65], which is isolated from cells by means of its detergent resistance. Furthermore, the destruction of rafts prevents the internalization of *P. aeruginosa* into epithelial cells and cellular apoptosis in vitro and in vivo in respiratory epithelial cells after pulmonary infection [37, 65]. The results of these studies further indicate the importance of distinct membrane domains for the infection of mammalian cells with *P. aeruginosa*.

A recent study by Yu and associates confirmed the role of acid sphingomyelinase in *Cftr*-deficient cells and in the lungs of *Cftr*-deficient mice [64]. The results of these studies confirmed the previous findings that infecting

cells with *P. aeruginosa* triggers an activation of acid sphingomyelinase and a release of ceramide. The results also demonstrate that the activation of acid sphingomyelinase and the formation of ceramide do not occur in cells lacking *Cftr* upon infection with *P. aeruginosa* [64]. The defect of acid sphingomyelinase activation in *Cftr*-deficient cells may be caused by the altered pH in intracellular vesicles in *Cftr*-deficient cells, as discussed below [11, 69-71]. Furthermore, the fact that the release of IL-8 is higher in *Cftr*-deficient cells infected with *P. aeruginosa* than in wild-type control cells is associated with the defect in activating acid sphingomyelinase and is partially corrected by adding exogenous acid sphingomyelinase [11, 64]. Cells lacking *Cftr* or transfected with small interfering RNA (siRNA), which suppresses the expression of acid sphingomyelinase, also demonstrate a failure to internalize *P. aeruginosa* [64]. Finally, *Cftr*-deficient cells fail to respond to *P. aeruginosa* by inducing apoptosis upon lung infection [11, 64, 72]. Adding exogenous sphingomyelinase to *Cftr*-deficient cells restores apoptosis [64]. The results of these studies confirm the importance of acid sphingomyelinase in infection with *P. aeruginosa*; they also demonstrate that at least some features of *Cftr* are linked to a failure of *Cftr*-deficient cells to acutely activate acid sphingomyelinase and to release ceramide upon acute infection.

Ceramide in cystic fibrosis

We have recently demonstrated that ceramide accumulates in bronchial epithelial cells and alveolar macrophages of *Cftr*-deficient mice (Fig. 2) [11, 73]. The results of biochemical, fluorescence microscopy, and mass spectrometry studies have demonstrated an age-dependent accumulation of ceramide in the lungs of various *Cftr*-deficient mouse strains, in particular in the

epithelial cells of large and small bronchi and macrophages. It is important to note that ceramide accumulation occurs without any pulmonary infection. Pulmonary ceramide accumulation has been observed not only in two strains of *Cftr*-deficient mice [11, 73] but also in ciliated nasal epithelial cells and in lung specimens from cystic fibrosis patients [11, 73]. However, results of studies by Radzioch and colleagues suggest that ceramide concentrations are decreased in cystic fibrosis patients [74].

To reconcile these two findings, it is important to clearly define the term *ceramide*. In the studies by Radzioch and colleagues, ceramide was extracted from cells, separated by thin-layer chromatography, and determined by an ELISA assay using the monoclonal 15B4 anti-ceramide antibody. This antibody detects not only unsaturated ceramides, which are usually referred to by the term *ceramide*, but also dihydroceramides that are intermediates of the synthesis of unsaturated ceramides. Furthermore, the studies by Radzioch and colleagues demonstrated a normalization of “ceramide” concentrations by treatment with fenretinide, a drug known to inhibit the activity of dihydroceramide desaturase and to stimulate the activity of dihydroceramide synthase (Fig. 1). Thus, it is very likely that the methods used by Radzioch and colleagues predominantly determined the concentration of dihydroceramide.

Our studies, on the other hand, determined the concentration of unsaturated ceramides with mass spectrometry and with either the DAG-kinase assay or fluorescence microscopy using the monoclonal S58-9 anti-ceramide antibody [11, 73]; neither the DAG-kinase assay nor the S58-9 anti-ceramide antibody detects dihydroceramides. Mass spectrometry clearly differentiates unsaturated from saturated ceramides. It is tempting to speculate that the levels of ceramide and dihydroceramide are inversely correlated. Although it is clear that unsaturated ceramides play many biological roles [for reviews see 15, 22, 23], the role of dihydroceramides in cell signaling remains to be determined, although a role in autophagy has been suggested [75]. It is very likely that unsaturated ceramides and dihydroceramide are differentially regulated in cystic fibrosis and that an increase in the end-product, unsaturated ceramide, may result in a compensatory decrease in the concentration of dihydroceramide. In our studies, we use the term *ceramide* to refer exclusively to unsaturated ceramides.

Ceramide is released from sphingomyelin by the activity of sphingomyelinases, is synthesized de novo, or both. The results of genetic and pharmacological studies

Fig. 3. Ceramide accumulation in cystic fibrosis as therapeutic target. A defect in the *Cftr* gene results in an accumulation of ceramide in bronchial epithelial cells. Genetic or pharmacological inhibition of acid sphingomyelinase results in normalization of pulmonary ceramide levels and thereby reduces inflammation, improves mucociliary clearance, and prevents infection with *P. aeruginosa* [11].

indicate that the inhibition of acid sphingomyelinase activity is sufficient to nearly normalize ceramide concentrations in the lungs of *Cftr*-deficient mice (Fig. 3). Genetic inhibition of acid sphingomyelinase activity is achieved by crossing *Cftr*-deficient mice with acid sphingomyelinase-deficient mice, thereby producing mice that are deficient in *Cftr* and heterozygous for acid sphingomyelinase [11]. Pharmacological inhibition of acid sphingomyelinase is achieved by treating *Cftr*-deficient mice with amitriptyline, a functional acid sphingomyelinase inhibitor [11, 76, 77]. Either genetic or pharmacological inhibition of acid sphingomyelinase reduces acid sphingomyelinase activity by 50% to 70%, an amount sufficient to normalize ceramide concentrations in the lungs of cystic fibrosis mice.

Increased ceramide concentrations in bronchial epithelial cells and lung macrophages of *Cftr*-deficient mice are involved in several pulmonary alterations that are typical for cystic fibrosis, such as inflammation, reduced mucociliary clearance, and high susceptibility to pulmonary infection with *P. aeruginosa*.

(i) *Inflammation.* The results of several studies in recent years have suggested an imbalance between proinflammatory and anti-inflammatory cytokines in the airways of cystic fibrosis patients and mice even prior to any infection [11, 78–80]. In *Cftr*-deficient cells, the increased formation of pro-inflammatory mediators, such as IL-1 or IL-8/KC, and the simultaneous downregulation of anti-inflammatory cytokines, such as IL-10 [11, 78–83], may result in chronic aseptic inflammation of the lung, tissue destruction, reduction of mucociliary clearance, and high susceptibility to infection with *P. aeruginosa*.

Fig. 4. Role of ceramide in cystic fibrosis and acute *P. aeruginosa* infections. Infection of wild-type cells with *P. aeruginosa* results in activation of the acid sphingomyelinase and acute release of ceramide. Ceramide mediates internalization of the pathogen into epithelial cells, focal induction of cell death and a controlled release of cytokines. These events are absent in mice lacking the acid sphingomyelinase and these mice are unable to control and to eliminate the infection. *Cftr*-deficient epithelial cells show an increased ceramide concentration even prior to any infection. Increased ceramide concentrations in epithelial cells triggers cell death and the deposition of DNA in airways and induces chronic inflammation. The reduced mucociliary clearance of pathogens results in an increased sensitivity of *Cftr*-deficient mice to pulmonary *P. aeruginosa* infection.

Normalizing ceramide concentrations in the lungs by inhibiting acid sphingomyelinase also normalizes the concentrations of the proinflammatory mediators IL-1 and IL-8, as well as the number of macrophages and neutrophils, in the lungs of *Cftr*-deficient mice [11]. Ceramide has been shown to activate several transcription factors, including NFκB [84, 85]; which has also been reported to be upregulated in cells lacking *Cftr* [86, 87]. Thus, it is possible that increased ceramide concentrations mediate the upregulation of proinflammatory mediators by stimulating transcription factors in *Cftr*-deficient cells.

(ii) *Mucociliary clearance.* Analysis of cell death in the bronchi of cystic fibrosis mice demonstrated that the death rate of bronchial epithelial cells is higher in *Cftr*-deficient mice than in normal mice [11]. Increased cell death and the deposition of dead cells in the bronchial lumen results in the formation of DNA-containing plugs and deposits in the bronchi. Because DNA is very viscous, these cell remnants may decrease mucociliary clearance. Pharmacological or genetic inhibition of acid sphingomyelinase and, thus, normalization of ceramide concentrations in the lungs of cystic fibrosis mice normalize the death of bronchial epithelial cells in the respiratory tract of *Cftr*-deficient mice. At present, the mechanisms by which increased ceramide concentrations in epithelial cells trigger cell death are unknown. DNA deposits may not only decrease mucociliary clearance but also facilitate *P. aeruginosa* adherence in and infection of the respiratory tract. Thus, ceramide-mediated cell death may play multiple roles in the pathogenesis of cystic fibrosis.

(iii) *Infection.* Infecting *Cftr*-deficient mice intranasally with *P. aeruginosa* demonstrated that these mice are much more susceptible to the pathogen than

are normal mice [11]. Inhibiting acid sphingomyelinase activity by approximately 50% normalizes ceramide concentrations in the lungs of cystic fibrosis mice. It also normalizes the susceptibility of these mice to infection with *P. aeruginosa* and reduces the bacterial load in the lung by at least a factor of 100 compared to the bacterial load of untreated mice. It should be noted that a complete genetic deficiency of acid sphingomyelinase also results in increased sensitivity of the mice to *P. aeruginosa*, although by a completely unrelated mechanism: Although the initial number of bacteria in the lungs of *Smpd1*^{-/-} mice after intranasal infection is very similar to that in the lungs of wild-type mice, the *Smpd1*^{-/-} mice cannot remove the bacteria once the infection has been established [37]. In contrast, *Cftr*-deficient mice exhibit a defect in the early phase of the infection period, and much higher numbers of bacteria reach the lungs of these mice immediately after infection than is the case in wild-type mice [11]. These concepts are displayed in Fig. 4.

Treatment of cystic fibrosis by inhibition of acid sphingomyelinase

Recent studies have identified a variety of inhibitors of acid sphingomyelinase, all of which inhibit the enzyme by the following mechanism: Acid sphingomyelinase binds to the membrane of lysosomes and secretory lysosomes, and this binding protects acid sphingomyelinase from proteolytic inactivation [76, 77]. Weak bases, such as amitriptyline, accumulate in lysosomes, are protonated within these acidic compartments, and, thus, are concentrated in these organelles. The drugs bind to the membrane and displace acid sphingomyelinase from the

membrane; this displacement results in proteolytic degradation of the enzyme. The lipophilic ring structures present in these functional acid sphingomyelinase inhibitors seem to integrate into the lysosomal membrane, whereas the cationic amino end-group of the aliphatic chain displaces acid sphingomyelinase from the membrane.

The results of murine studies suggest that inhibition of acid sphingomyelinase may be a novel therapeutic strategy for treating patients with cystic fibrosis. Initial clinical findings from a small study in which patients were treated with amitriptyline confirm this notion and indicate that treatment with amitriptyline may improve lung function in cystic fibrosis patients [73, 88] (Fig. 3). These studies demonstrated that the use of inhibitors of the acid sphingomyelinase is safe in patients with cystic fibrosis. The studies further indicate that even low doses of the drug given systemically to patients with cystic fibrosis improves the lung function as determined by measurements of the forced expiratory volume in the

1st second (FEV1). In addition, several cystic fibrosis patients treated for several years with amitriptyline showed a continuous increase of lung function. Finally, the drug also normalized the body weight of the patients and seemed to reduce the number of upper respiratory tract infections. However, since these the first in men studies to inhibit the acid sphingomyelinase with amitriptyline to treat cystic fibrosis, it must be pointed out that the number of patients included in these studies is still too low to make a final conclusion about the positive effects of amitriptyline in cystic fibrosis and further, larger clinical studies are required.

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