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Anti-inflammatory action of lipid nanocarrier-delivered myriocin: therapeutic potential in cystic fibrosis

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ABSTRACT

Background: Sphingolipids take part in immune response and can initiate and/or sustain inflammation. Various inflammatory diseases have been associated with increased ceramide content, and pharmacological reduction of ceramide diminishes inflammation damage *in vivo*. Inflammation and susceptibility to microbial infection are two elements in a vicious circle. Recently, sphingolipid metabolism inhibitors were used to reduce infection. Cystic fibrosis (CF) is characterized by a hyper-inflammation and an excessive innate immune response, which fails to evolve into adaptive immunity and to eradicate infection. Chronic infections result in lung damage and patient morbidity. Notably, ceramide content in mucosa airways is higher in CF mouse models and in patients than in control mice or healthy subjects.
Methods: The therapeutic potential of myriocin, an inhibitor of the sphingolipid *de novo* synthesis rate limiting enzyme (Serine Palmitoyl Transferase, SPT),was investigated in CF cells and mice models.
Results: We treated CF human respiratory epithelial cells with myriocin, This treatment resulted in reduced basal, as well as TNFα-stimulated, inflammation. In turn, TNFα induced an increase in SPT in these cells, linking *de novo* synthesis of ceramide to inflammation. Furthermore, myriocin-loaded nanocarrier, injected intratrachea prior to *P. aeruginosa* challenge, enabled a significant reduction of lung infection and reduced inflammation.
Conclusions: The presented data suggest that *de novo* ceramide synthesis is constitutively enhanced in CF mucosa and that it can be envisaged as pharmacological target for modulating inflammation and restoring effective innate immunity against acute infection.
General significance: Myriocin stands as a powerful immunomodulatory agent for inflammatory and infectious diseases.

1. Introduction

Sphingolipids (SPL) are a broad class of membrane components and signaling mediators involved in cell survival and function. It is becoming increasingly apparent that SPL take part to inflammation and to host innate response upon infection [1–3]. The sphingolipid ceramide is highly effective in the activation of inflammation related transcription factors, such as NF-kB [4] and AP1 [5] and in receptors clustering/signaling upon inflammatory stimula [6]. Patients suffering from chronic inflammation such as irritable bowel syndrome [7], emphysema lung injury and chronic obstructive pulmonary disease [8–10], exhibit increased levels of mucosal ceramide as compared to controls. A vicious loop relates the excessive inflammation to the susceptibility to microbial infection. Hyper-inflammation associates with the inability to clear infections at their early stage, as well as to mature the adaptive immune response, thus allowing the chronic establishment of pathogens communities. Ceramide accumulation may derive from increased synthesis *de novo* or from altered metabolism of complex sphingolipids such as sphingomyelin or glycosphingolipids. The role of sphingomyelinases, neutral and acidic, in inflammation has been extensively investigated [11,12]. The hydrolysis of plasma-membrane sphingomyelin is responsible for ceramide-rich membrane platforms formation and required for signal transduction of inflammatory stimula, such as TNFα, ILβ, INFγ [6,8,13,14], increased vascular permeability [15,16] as well as for the internalization of microorganisms [13,17]. On the other side, only a few reports underscore the involvement of *de novo* synthesis of ceramide in the inflammatory responses [10,18–20]. Thus, emphysema

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lung damage [10] and immune-reactivity in murine dorsal horn of the lumbar spinal cord were reduced by Fumonisin B1, an inhibitor of ceramide synthase [18]. Furthermore, mice feeding with myriocin (Myr), an inhibitor of serine palmitoyl transferase (SPT), decreased radiation-induced inflammation and fibrosis [21]. A recent finding demonstrated that reduced *de novo* ceramide synthesis by fenretinide, associated with the increase of its precursor dihydroceramide, impairs bacterial infection in macrophages [19].

Cystic fibrosis (CF) is an inherited autosomal recessive disease caused by CF transmembrane conductance regulator (CFTR) mutations. CF patients develop mucus viscosity, impaired mucociliary transport, hyper-inflammation and severe alteration of all mucosal functions with lung disease [22,23] and chronic opportunistic infections (mainly *Pseudomonas aeruginosa*, *Bhurkolderia cepacia complex*, and *Staphylococcus aureus)* remaining the main cause of morbidity and mortality [24,25]. Inflammation is an independent risk factor for CF disease progression. Even the uninfected CF lungs of foetuses or two years old infants, develop a pathological inflammatory condition [26–29], confirming the severe immune alteration of mucosa in the respiratory tract.

In mouse models of CF, an age-related accumulation of ceramide in respiratory epithelium was associated to the pathological inflammatory state and infection susceptibility. Sphingomyelinase [9,30,31], or ceramide synthase [9] inhibition, or fenretinide, an inhibitor of ceramide formation from its precursor dihydroceramide [32], reduced inflammation and infection in CF mice.

Similarly, in CF patients, increased ceramide content was found in nasal respiratory epithelium and lungs [30,33,34]. Amitriptyline, an inhibitor of acid sphingomyelinase used in a phase II study, reduced ceramide levels in the respiratory epithelial cells of treated patients and this was accompanied by a significant increase in lung function [30,31].

In spite of the increased ceramide mass in CF mucosa, there is no evidence on what is the rate of ceramide synthesis *versus* its release from membrane sphingomyelin. We hypothesized that preventing *de novo* sphingolipid synthesis with Myr could reduce excessive lung inflammation in CF and allow an efficient innate response to acute infection. In this article we demonstrated that Myr reduces IL-8 and IL-6 release in human CF respiratory epithelium. Given the hydrophobicity of the compound, we sought to deliver Myr *in vivo*, in murine airways, by means of solid lipid nanoparticles (SLN) [35]. We demonstrated that Myr-loaded SLN are able to reduce inflammation and infection in CF mice lung.

2. Materials and methods

2.1. Reagents and antibodies

Myr was purchased from Fermentek LTD (Israel), MTT and bovine serum albumin (BSA) were from Sigma-Aldrich (US). LHC Basal, LHC-8 without gentamicin culture media (Gibco, US), Penicillin and streptomycin (Invitrogen) were purchased from Life Technologies Italia (Italy). Fetal bovine serum (FBS) and the chemiluminescence system LiteAbLot were purchased from EuroClone Life Science Division (Italy). Human Fibronectin and Bovine Collagen were from Becton-Dickinson Italia (Italy). Human and mouse IL-8, IL-6 mini EDK and human TNF-α were from Peprotech (UK). The synthetic oligonucleotides used in this study were purchased from M-Medical (Italy). All reagents were of the maximal available purity degree.

2.2. Myriocin stock solution preparation

Myr powder was weighted and dissolved in DMSO by warming up at 37 °C, to a final concentration of 2 mM. Solution was sterile filtered (0.22 μm pore diameter, Nalgene) and stored at 4 °C until used. This stock solution was diluted in medium for cell treatment (final treatment concentration: 10 μM) and in sterile saline for animal treatments (final treatment concentration: 420 μM, equal to 11.95 μg of Myr *per* mouse).

2.3. Myriocin loaded-solid lipid nanoparticles (SLN) used for mice treatment

Treatment of mice with Myr was achieved by using Myr-loaded SLNs (Nanovector srl, Italy) prepared as previously described [35]. SLN loaded with drug were measured for Myr content and a 1 mM Myr-SLN stock solution was prepared. This solution was diluted 1:12 in sterile saline and 75 μl (1.7 μg of Myr and 8% SLN) were used for each mouse administration in the airways.

3. Cell lines and treatments

IB3-1 cells, an adeno-associated virus-transformed human bronchial epithelial cell line derived from a CF patient (ΔF508/W1282X) and its isogenic C38 cells, corrected by insertion of CFTR, have been both obtained from LGC Promochem (US) and kindly provided by the Cystic Fibrosis animal Core Facility (CFaCore, San Raffaele Hospital, Milan, Italy). Cells were grown in LHC-8 media supplemented with 5% FBS. Both culture flasks and plates were coated with a solution of LHC-basal medium containing 35 μg/ml bovine collagen, 1 μg/ml bovine serum albumin and 10 μg/ml human fibronectin as described [36]. For experiments, cells were seeded in 6 multi-wells plate or 100 mm petri dishes at 3×10^5 and 2×10^6 cells/plate respectively. Twenty four h after seeding, when cells reached about 60% confluence, medium was replaced with fresh one, containing either Myr (10 μM) or vehicle (DMSO). Eight h after Myr treatment, human TNFα (20 ng/ml) was added to both treated and untreated cells. Incubation proceeded for further 18 h and then samples were collected for analyses.

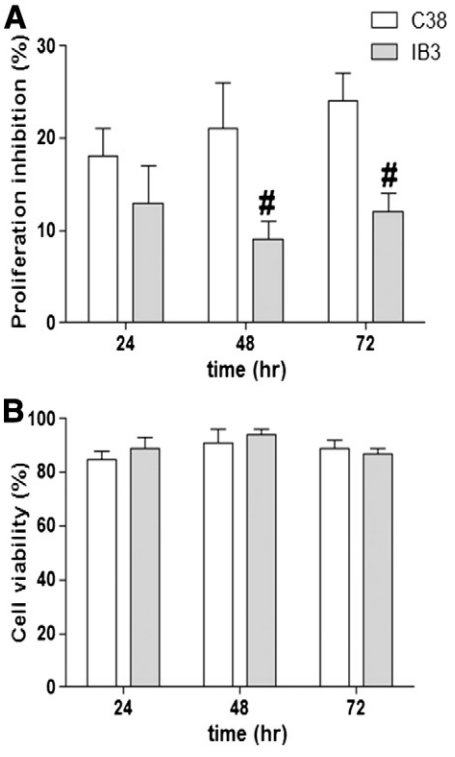

Fig. 1. Myriocin differently affects proliferation of C38 *vs* IB3 cells. Inhibitory effect of Myr (10 μM) on IB3 and C38 cells proliferation at 24, 48, 72 h, expressed as percentage of proliferation inhibition in treated *vs* control (**A**). Significance was evaluated by one-way ANOVA, #, vs C38 cells ($P < 0{,}005$, Bonferroni post-test). Cell viability, calculated as percentage of alive vs total cells in IB3 and C38 cells treated with Myr (10 μM) for 24, 48, 72 h (B).