Corticosteroids protect infected cells against mycobacterial killing in vitro

Hasan Tükenmez a, Isabel Edström a, Sadaf Kalsum b, Clara Braian b, Ramesh Ummannic, Stina Berglund Fickd, Charlotta Sundine, Maria Lerm b, Mikael Elofsson e, Christer Larsson f, *

a Department of Molecular Biology, Umeå University, SE-901 87, Umeå, Sweden
b Department of Clinical and Experimental Medicine, Linköping University, SE-581 83, Linköping, Sweden
c Department of Applied Biology, CSIR-Indian Institute of Chemical Technology (CSIR-IICT), Tarnaka, Hyderabad, 500007, Telangana, India
d Chemical Biology Consortium Sweden (CBCS), Department of Chemistry, Umeå University, SE-901 87, Umeå, Sweden
e Department of Chemistry, Umeå University, SE-901 87, Umeå, Sweden
f Infectious Diseases Clinic, Umeå University Hospital, SE-901 85, Umeå, Sweden

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Abstract
The effect of corticosteroids on human physiology is complex and their use in tuberculosis patients remains controversial. In a high-throughput screening approach designed to discover virulence inhibitors, several corticosteroids were found to prevent cytolysis of fibroblasts infected with mycobacteria. Further experiments with Mycobacterium tuberculosis showed anti-cytolytic activity in the 10 nM range, but no effect on bacterial growth or survival in the absence of host cells at 20 μM. The results from a panel of corticosteroids with various affinities to the glucocorticoid- and mineralocorticoid receptors indicate that the inhibition of cytolysis most likely is mediated through the glucocorticoid receptor. Using live-imaging of M. tuberculosis-infected human monocyte-derived macrophages, we also show that corticosteroids to some extent control intracellular bacteria. In vitro systems with reduced complexity are to further study and understand the interactions between bacterial infection, immune defense and cell signaling.

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1. Introduction

Tuberculosis (TB) is the leading cause of death from a single infectious agent and one of the top 10 causes of death worldwide. WHO estimates that about 10 million people develop TB disease each year [1]. One of the hallmarks of TB is its ability to cause latent infection. In such cases, the infected person has no symptoms, is not contagious, but is at risk for reactivation. The mechanisms of reactivation are poorly understood, but it is widely believed that patients with latent TB treated with immunosuppressive agents such as corticosteroids are at high risk of reactivation and this has been demonstrated in several case-control studies [2,3]. Agents with immunosuppressive activity are therefore generally contraindicated in TB patients.

Our team recently performed a high-throughput screening campaign to discover substances capable of preventing cytolysis of fibroblast cells upon mycobacterial infection [4]. With this strategy, we expected to discover classic antibacterials with bacteriostatic and bactericidal effects, virulence inhibitors with bacterial targets and substances with impact on the host cell in line with an immunomodulatory mode of action. The screen resulted in 49 substances which met the selection criteria, but among these 38 were excluded as known antibiotics, or for having undesirable properties. Among the substances initially excluded for having undesirable properties were five corticosteroids (data not shown).

Although initially rejected for having adverse effects on TB, the corticosteroids had caught our interest and several reports in the literature indicate that corticosteroids may have a beneficial effect for TB outcome. In a joint publication from the American Thoracic Society, Centers for Disease Control and Prevention and Infectious Diseases Society of America, corticosteroid treatment is strongly recommended in the treatment of TB pericarditis and TB of the central nervous system, but not recommended for other
Corticosteroids are widely used in treatment of allergy, asthma, stasis and many other physiological processes. Synthetic glucocorticoids, such as hydrocortisone, dexamethasone and prednisone, are best known for their strong anti-inflammatory and immune modulatory activity, but they are also important in fetal development, metabolism, body fluid homeostasis and many other physiological processes. Synthetic glucocorticoids are widely used in treatment of allergy, asthma, stasis and many other physiological processes. Synthetic glucocorticoids, its anti-inflammatory activity is mediated mainly by inhibition of NF-κB and c-Jun-Fos and by induction of proteins such as MAPK phosphatase 1, IκB and annexin I among others, as reviewed in Ref. [7]. The mechanisms of the GR and how it can possess both pro- and anti-inflammatory activity is not fully understood.

Corticosteroids have vastly different effects in different cell types. While glucocorticoids can induce apoptosis in many cell types throughout the body, other exhibit an anti-apoptotic response to glucocorticoid signaling [8]. In macrophages, glucocorticoids have immunostimulatory effects [9], and act as a potent anti-apoptotic in primary human fibroblasts whereas it is pro-apoptotic in hematopoietic cells. The anti-apoptotic effect by dexamethasone is attributed to a combination of phosphoinositide 3-kinase/Akt signaling and involvement of the BCL2 family protein BCL-XL [10].

2. Materials and methods

2.1. Cell culture

Human lung fibroblast cell line MRC-5 (ATCC® CCL-171™) was maintained and expanded in Advanced Dulbecco’s Modified Eagle Medium (A-DMEM) (Life technologies) supplemented with 5% new-born calf serum and 0.3% (w/v) l-glutamine at 37 °C with 5% CO2 until 80% confluent.

Wild-type Mycobacterium marinum M (ATCC BAA-535™) and a RD1 knockout with M. marinum M background (a kind gift from Fredric Carlsson, Lund university) were grown in 7H9 medium (BD Difco) supplemented with 0.05% Tween80 and 10% ADS (0.5% albumin, 0.2% dextrose and 0.085% saline) as standing cultures at 30 °C. M. tuberculosis H37Rv, H37Rv harboring the pCherry3 plasmid carrying mCherry gene under a constitutive promoter (Psynn) [14] and H37Ra were also grown in 7H9 medium supplemented with 0.05–0.10% Tween80 and 10% ADS as standing cultures at 37 °C.

2.2. Compounds

A chemical library of 28,000 compounds provided as stock solutions in dimethyl sulfoxide (DMSO) from the Laboratories for Chemical Biology Umeå and Chemical Biology Consortium Sweden library was screened at 20 μM to identify hits that would prevent M. marinum-induced cytolysis of MRC-5 fibroblasts as described previously [4]. Budesonide (Cayman chemical), Fluorocortisone acetate, deoxycorticosterone acetate, dexamethasone (MP Biomedicals), flumethasone, flunisolide (Santa Cruz), hydrocortisone, aldosterone, betamethasone, triamcinolone acetate (Acras Organics), fluorocinolone acetonide (abcam) and desonide (Sigma-Aldrich) were dissolved in DMSO to 10 mM and stored at 4 °C until used in experiments. Working solutions of isoniadiz (Fluka) and rifampicin (G Biosciences) were freshly prepared in DMSO for each experiment.

2.3. Activity of corticosteroids on M. tuberculosis

Twelve corticosteroids were assayed at concentrations ranging from 0.001 μM to 0.5 μM on MRC-5 fibroblast cells infected with M. tuberculosis H37Rv in 96-well plates as described previously [4]. Corticosteroid impact on MRC-5 cell viability was plotted as bar graphs based on average and standard deviation of triplicates. In order to determine if corticosteroids have bactericidal or bacteriostatic activity, bacterial growth and survival was assayed with corticosteroid concentrations up to 20 μM in 7H9 media as described in Ref. [4].

2.4. Preparation of human monocyte-derived macrophages (hMDMs)

Peripheral blood mononuclear cells (PBMCs) were isolated from buffy coats obtained from healthy volunteers who gave their written informed consent for the use of their blood for scientific purposes (Linköping University Hospital blood bank, Linköping, Sweden) as described in Ref. [15]. PBMC isolation was performed using LymphoPrep (Axis-Shield), according to the instructions from the manufacturer. The mononuclear cells were seeded in culture flasks and later differentiated into hMDMs as previously described [16].

2.5. Infection of hMDMs monitored by IncuCyte live cell microscopy

The hMDMs obtained from two different donors were washed, trypsinated and resuspended in DMEM, (Gibco), supplemented with 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2 mM l-Glutamine (Gibco) and 10% non-heat inactivated human serum pooled from 5 donors (Linköping University Hospital). The hMDMs were seeded in a 384-well plate (8000 cells/well) and infected with M. tuberculosis H37Rv:pCherry3 reporter strain at a multiplicity of infection (MOI) of 1:1 in the presence of 0.5 μM of corticosteroid. To control wells, 0.4% DMSO or 0.1 μg/ml isoniaizid or 0.1 μg/ml rifampicin was added. The plate was incubated at 37 °C with 5% CO2. At different time points (day 1, 2, 3, 4 and 5) images from each well were acquired with an IncuCyte live-cell microscope (Essen Bioscience). Custom analysis scripts within the IncuCyte Software were used to measure fluorescence signal from M. tuberculosis H37Rv:pCherry3 reporter strain. In this custom
concentrations of (lower dotted line). The bar graph is plotted based on average and standard deviation values obtained from independent triplicates. Corticosteroid concentrations are in μM. The graph might be beneficial for reactivating latent TB [2,3,17,18]. However, recent clinical studies suggest that usage of corticosteroids during active TB infection is difficult to treat and new drugs and treatment strategies are much needed.

In a previous study, we therefore screened 28,000 compounds to identify substances with anti-cytolytic activity in MRC-5 fibroblasts infected with M. marinum as well as M. tuberculosis H37Rv without having any bactericidal or bacteriostatic effect [4]. However, after the initial screening, 38 hits had been discarded as already known antibacterials or assumed not to be suitable for TB treatment. Amongst these initially discarded hits, one group of substances caught our attention – the corticosteroids. Budesonide, fludrocortisone acetate, flumethasone, flunisolide and hydrocortisone base were all preventing cytolsis of MRC-5 cells infected with M. marinum (data not shown). These hits were discarded in our earlier publication since corticosteroids generally are regarded as a risk factor for reactivating latent TB [2,3,17,18]. However, recent clinical studies suggest that usage of corticosteroids during active TB infection might be beneficial [5,6].

We decided to further investigate the effects of corticosteroids in our fibroblast infection assay by evaluating the five initial corticosteroid screening hits as well as additional seven corticosteroid compounds in M. tuberculosis H37Rv infected MRC-5 fibroblasts and comparing the MRC-5 viability in the end of a two-day infection. All corticosteroids except deoxycorticosterone acetate and aldosterone prevented cytolysis of the MRC-5 cells at concentration range from 0.001 μM to 0.1 μM (Fig. 1). Interestingly, deoxycorticosterone acetate and aldosterone are potent activators of the mineralocorticoid receptor but have little or no glucocorticoid activity [13,19]. In contrast, all corticosteroids with anticytolytic activity have glucocorticoid activity as well as some also have additional mineralocorticoid activity [13,19]. These results suggest that the glucocorticoid anti-cytolytic activity observed is mediated by the GR rather than the mineralocorticoid receptor.

Since fibroblasts are not natural host cells of M. tuberculosis, we decided to test the effect of corticosteroids in a biologically more relevant intracellular infection model. We infected hMDMs obtained from two different donors with 1 MOI of M. tuberculosis H37Rv:pCherry3 and followed bacterial growth from day 1 to day 5 using an IncuCyte live-cell microscope (Essen Bioscience). Despite the fact that none of these corticosteroids were bacteriostatic or bactericidal up to 20 μM in 7H9 medium (Supplementary Fig. 1), they significantly reduced the intracellular bacterial growth in hMDMs cells at 0.5 μM (Fig. 2). Corticosteroids with glucocorticoid activity significantly reduced bacterial growth in hMDMs obtained from both donors (Fig. 2), whereas also corticosteroids with

Fig. 1. Corticosteroids rescue MRC-5 fibroblasts infected by M. tuberculosis from cytolysis. Viability of MRC-5 fibroblasts was determined by the resazurin conversion assay after two days of infection with M. tuberculosis in the presence of various concentrations of corticosteroids. The viability of MRC-5 fibroblasts exposed to avirulent M. tuberculosis H37Ra was set to 100% (upper dotted line) and the rest of the data was normalized accordingly. Virulent H37Rv untreated control results in 24% viability compared to H37Ra exposure (lower dotted line). The bar graph is plotted based on average and standard deviation values obtained from independent triplicates. Corticosteroid concentrations are in μM and concentrations of first-line antibiotics isoniazid and rifampicin controls are in μg/ml.
mineralocorticoid activity but low glucocorticoid activity (deoxycorticosterone acetate and aldosterone) seemed to have some activity in cells from donor 1 (Fig. 2). This is not very surprising since numerous single nucleotide polymorphisms in a number of genes are well known to determine the individual response to inhaled corticosteroids. For instance, asthma treatment with corticosteroids has a large inter-individual variability with numerous patients not responding to the treatment at all [20,21]. Moreover, 11β-hydroxylation of deoxycorticosterone produces a corticosteroid with glucocorticoid activity [12], and aldosterone can to some degree bind the GR resulting in GR-mediated cellular response [11].

The corticosteroid system itself is very complex and the level of complexity increases drastically with TB infection. A massive immune response, inflammation, pharmacokinetic interactions between corticosteroids and TB drugs as reviewed in Ref. [22], as well as rifampicin itself activating the GR [23], forms an overwhelming complexity.

We cannot form an opinion whether corticosteroid use in TB is beneficial or not based on the experiments presented and this was never the aim of the study. Instead, we attempted to simplify the...
complexity, and by studying the effect of corticosteroids on infected cells in vitro we show that glucocorticoids protect against mycobacterial killing. We believe simplified model experiments will be key to decipher the immense complexity of corticosteroid effects on TB infection.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrc.2019.02.044.

Transparency document

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