ELSEVIER

International Immunopharmacology 7 (2007) 230 – 240

International Immunopharmacology

www.elsevier.com/locate/intimp

Highly homologous *Mycobacterium tuberculosis* chaperonin 60 proteins with differential CD14 dependencies stimulate cytokine production by human monocytes through cooperative activation of p38 and ERK1/2 mitogen-activated protein kinases

Jo C. Lewthwaite [a,d], Claire E. Clarkin [d], Anthony R.M. Coates [b], Stephen Poole [c], Rachel A. Lawrence [d], Caroline P.D. Wheeler-Jones [d], Andrew A. Pitsillides [d], Mahavir Singh [e], Brian Henderson [a,*]

[a] *Division of Microbial Diseases, UCL Eastman Dental Institute, University College London, UK*
[b] *Department of Medical Microbiology, St George's Hospital Medical School, Tooting, London, UK*
[c] *Division of Endocrinology, National Institute for Biological Standards and Control, Potters Bar, Herts, UK*
[d] *Department of Veterinary Basic Sciences, Royal Veterinary College, London, UK*
[e] *Department of Biochemistry, TU-Braunschweig, c/o GBF, and Lionex GmbH, Braunschweig, Germany*

Received 24 January 2006; received in revised form 3 August 2006; accepted 11 October 2006

Abstract

Tuberculosis is a chronic inflammatory and destructive disease caused by infection with *Mycobacterium tuberculosis*. We have previously shown that the mycobacterial chaperonin (Cpn)60.1 and 60.2 proteins stimulate human monocytes to secrete pro-inflammatory cytokines.

Identification of the cellular mechanisms that contribute to the chronic inflammation characterised by myobacterial infection is therefore of potential therapeutic benefit. In the present study we have investigated the role of the extracellular signal-regulated (ERK1/2) and p38 mitogen-activated protein kinase (MAPK) families in Cpn60-induced cytokine synthesis, and have compared the effects of the bacterial proteins with those of lipopolysaccharide (LPS). Exposure to Cpn60.1, Cpn60.2 or LPS enhanced ERK1/2 activation with increases in phosphorylation evident between 10 and 30 min and maximal after 60–90 min stimulation. Phosphorylation of ERK1/2 in Cpn60-stimulated monocytes was maintained whereas ERK1/2 was rapidly dephosphorylated in LPS-stimulated cells. Exposure to the chaperonins also caused rapid activation of $p38^{mapk}$ with kinetics of phosphorylation comparable to those observed in response to LPS. Selective inhibitors of $p38^{mapk}$ (SB203580) or of MEK1/2, the direct upstream activator of ERK1/2 (PD98059), reduced the synthesis of IL-1β, TNFα, IL-6 and IL-8 induced by either the chaperonins or LPS. Experiments in which cells were exposed to a combination of both inhibitors led to a nearly complete abrogation of agonist-induced cytokine synthesis. These results show that the $p38^{mapk}$ and ERK1/2 signalling pathways are important regulators of the cellular response to mycobacterial chaperonins and that these pathways cooperate to regulate pro-inflammatory cytokine production by human monocytes.

Keywords: Chaperonin; Monocyte; MAPK; Cytokine

* Corresponding author. Division of Microbial Diseases, UCL Eastman Dental Institute, University College London, 256 Gray's Inn Road, London WC1X 8LD, UK. Tel./fax: +44 20 7915 1190.
E-mail address: B.Henderson@eastman.ucl.ac.uk (B. Henderson).

1567-5769/$ - see front matter © 2006 Elsevier B.V. All rights reserved.
doi:10.1016/j.intimp.2006.10.005

1. Introduction

Tuberculosis is recognised as one of the major global threats to human health with an estimated 1.9 billion of the World's population being latently infected with *Mycobacterium tuberculosis* and with more than 3 million people dying each year of the disease [1,2]. Tuberculosis is a chronic inflammatory and destructive disease in which activation of myeloid cells plays a major role in pathology. It is therefore important to identify the mechanisms by which the causative agent, *M. tuberculosis*, produces an inflammatory response. Surprisingly, the number of inflammogenic components of *M. tuberculosis* identified to date is small: lipoarabinomannan [3], mannosylated phosphatidylinositol [3], trehalose dimycolate [4] and lipoproteins [5]. Other components of mycobacteria promote inflammation by inducing immune responsiveness. Perhaps the best characterised of these is heat shock protein (hsp) 65, one of the most potent immunogens produced by *M. tuberculosis* [6].

In addition to being an important immunogen, it has been established that hsp 65, which is now known as chaperonin (Cpn)60.2 [7], directly activates human monocytes [8]. *M. tuberculosis* is one of the small proportions of bacteria that contain more than one chaperonin 60 gene [7] with the two chaperonin 60 genes being known as *cpn* 60.2 (which encodes hsp 65) and *cpn* 60.1 which encodes a protein with significant homology to hsp 65 [9]. We have recently reported that both recombinant Cpn60 proteins of *M. tuberculosis* stimulate cytokine synthesis by cultured human monocytes [10]. However, in spite of the sequence homology, there was a significant difference in the potency and mechanism of action of these two proteins. Thus, blocking antibodies to CD14 did not modify the effects of Cpn60.2 on human monocyte cytokine synthesis, whereas these antibodies blocked the effects of Cpn60.1. Moreover, a peptide fragment (residues 191–213) from within Cpn60.1 was also found to activate monocytes in a CD14-dependent manner, whilst the equivalent peptide from Cpn60.2 was inactive [10].

Given that these homologous proteins appear to activate monocytes through different cellular receptors we hypothesised that they may utilise different intracellular signalling pathways to regulate cytokine synthesis. Cpn60.1 would be expected to activate monocytes by triggering signalling pathways similar, if not identical, to LPS since both bind to the CD14 receptor. In contrast, this hypothesis would predict that Cpn60.2 uses signalling systems distinct from LPS and Cpn60.1. In the present study, we have examined; (i) the effects of Cpn60.1 and Cpn60.2 on the activation of the extracellular signal-regulated kinase 1/2 (ERK1/2) and $p38^{mapk}$ families and compared these effects with those of LPS from *E. coli*. (ii) Using selective inhibitors we have determined the roles of the ERK1/2 and $p38^{mapk}$ pathways in regulating Cpn60- and LPS-induced synthesis of key pro-inflammatory cytokines. Our results indicate that *M. tuberculosis* Cpn60.1 and Cpn60.2 proteins activate ERK1/2 and $p38^{mapk}$ signalling pathways, and that cooperative activation of ERK1/2 and $p38^{mapk}$ by mycobacterial Cpn60 proteins is required for optimal pro-inflammatory cytokine production by human monocytes.

2. Materials and methods

2.1. Materials

M. tuberculosis cpn 60.1 and *cpn* 60.2 were cloned and the recombinant proteins expressed and purified as described previously [10]. The endotoxin content of the recombinant chaperonins was shown to be low by *Limulus* amoebocyte assay [10] and the effects of residual LPS were negated by the routine addition of 5 μg/ml polymyxin B (Bedford Laboratories, USA) in all assays. The LPS used to compare monocyte activation was the hexa-acylated molecule from *E. coli* (Difco).

2.2. Preparation, analysis and culture of human monocytes

Human peripheral blood monocytes were prepared using buffy coat blood from normal donors (obtained from the North London Blood Transfusion Centre, Colindale) by density gradient centrifugation and differential adherence as described [11]. This monocyte preparation contains residual B and T lymphocytes. Therefore in some experiments the monocytes were further purified by depletion of T and B cells using Dynabeads (Dynal). Adherent mononuclear cells were washed in PBS then recovered by scraping and resuspended in PBS supplemented with 0.1% BSA (Sigma) and incubated for 15 min at 4 °C, prior to incubation with the mouse anti-human CD2 and anti-human CD3 (Harlan Sera Lab) antibodies, plus anti-human CD19 antibodies (a kind gift of Deborah Hardie, University of Birmingham) for 30 min at 4 °C. Unbound antibodies were removed by washing in PBS/0.1% BSA and cells with bound antibodies were removed by mixing with sheep anti-mouse Dynabeads (Dynal, Liverpool, UK) for 45 min at 4 °C followed by exposure to a magnet. Selective depletion of T and B lymphocytes was monitored by labelling cells before and after purification with mouse anti-human CD2, CD3, CD14 and CD19 antibodies and by analysing the distribution of leukocyte subpopulations by flow cytometry using a Beckton Dickinson FacsScan flow cytometer.

2.3. Determination of monocyte activation by assay of cytokine synthesis

Adherent monocytes were incubated in RPMI 1640 containing 2% FCS and various concentrations of Cpn60