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Research paper

Corticosteroid effects on COPD alveolar macrophages: Dependency on cell culture methodology

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ABSTRACT

It is unclear whether cell culture methodology affects the corticosteroid sensitivity of chronic obstructive pulmonary disease (COPD) alveolar macrophages. We compared the effect of a short and a long isolation procedure on corticosteroid inhibition of lipopolysaccharide (LPS) stimulated cytokine release from COPD alveolar macrophages. We also investigated signalling pathways associated with macrophage activation during cell isolation. Macrophages cultured using a short isolation protocol released higher unstimulated levels of tumour necrosis factor (TNF)-α and chemokine C–X–C motif ligand (CXCL) 8; these macrophages were less sensitive to corticosteroid inhibition of LPS stimulated TNF-α and CXCL8 release when compared to a long isolation procedure. This was associated with increased p38 mitogen activated kinase (MAPK) activation. The p38 MAPK inhibitor, BIRB-796, significantly reduced unstimulated cytokine release. A key finding of this study was that both cell culture methods showed no difference in the corticosteroid sensitivity between COPD and control macrophages. We conclude that the culture of alveolar macrophages using a short isolation procedure alters cytokine production through p38 MAPK activation; this is associated with a change in corticosteroid sensitivity.

1. Introduction

Alveolar macrophages play a key role in host defence to inhaled microbes and particulate matter (Gordon and Read, 2002). Macrophages are professional phagocytes which engulf invading antigens while minimising tissue damage (Murray and Wynn, 2011). These cells also regulate the immune response through the secretion of cytokines, chemokines, and growth factors.

COPD is characterised by an abnormal inflammatory response to inhaled noxious particles, commonly cigarette smoke (www.goldcopd.org). This inflammatory response involves increased numbers of macrophages in the airways (Hogg et al., 2004); these macrophages play a central role in co-ordinating pulmonary inflammation, for example by the secretion of CXCL8 which is known to be increased in the lungs of COPD patients (Keatings et al., 1996) and is a potent neutrophil chemoattractant (Kaur and Singh, 2013).

Inhaled corticosteroids (ICS) are the first choice anti-inflammatory treatment for COPD; these drugs improve lung function and reduce exacerbation rates (Calverley et al., 2007). However, many COPD patients treated with ICS have persistent airway inflammation and recurrent exacerbations (Bourbeau et al., 2007; Soriano et al., 2007). The effects of corticosteroids have been investigated using COPD alveolar macrophages cultured in vitro; it has been reported that corticosteroids have a reduced effect on cytokine production from COPD compared to control macrophages, leading to the suggestion that COPD macrophages have acquired corticosteroid

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resistance (Culpitt et al., 2003; Cosio et al., 2004). In contrast, we have repetitively found that the effects of corticosteroids on cytokine production from COPD and control macrophages are similar (Armstrong et al., 2009, 2011; Southworth et al., 2012; Higham et al., 2013; Lea et al., 2013; Plumb et al., 2013). We also observed that corticosteroids have a relatively limited effect on the macrophage secretion of certain inflammatory mediators that are centrally involved in the pathophysiology of COPD, such as CXCL8.

Alveolar macrophages are commonly isolated for culture by plate adherence. The duration of plate adherence may cause cell activation; plate adherence for 1 h results in higher levels of spontaneous TNF-α, CXCL8, and interleukin (IL)-6 production compared to plate adherence for 24 h (Tomlinson et al., 2012). These results demonstrate that alveolar macrophages show an increased level of activation soon after isolation; this was also associated with a failure to demonstrate an incremental response to subsequent LPS stimulation. Plate adherence itself may be the cause of this acute cellular activation, but the saline flushing required to retrieve macrophages from the lungs may also cause cell activation by osmotic or mechanical stress (Denkert et al., 1998; Aikawa et al., 2002; Shiratsuchi and Basson, 2005). A longer duration of culture for cell isolation appears to allow initial cell activation, due to either plate adherence or saline flushing, to settle over time.

It appears that the activation state and LPS responsiveness of macrophages differ according to the time of stimulation after isolation (Tomlinson et al., 2012). This phenomenon has not been studied in COPD alveolar macrophages. It is important to understand whether methodological differences in protocols for COPD macrophage culture used in different studies could lead to different states of cell activation that change corticosteroid sensitivity.

The aim of this study was to investigate the effect of cell culture methodology on the corticosteroid sensitivity of COPD alveolar macrophages. The effect of corticosteroids on cytokine production from unstimulated and LPS stimulated macrophages isolated using different protocols was investigated. We also investigated changes in cell signalling pathways during different macrophage isolation protocols.

2. Methods

2.1. Study subjects

43 patients undergoing surgical resection for lung cancer were recruited (Table 1). COPD was diagnosed based on ≥10 pack years smoking history, typical symptoms and airflow obstruction. Controls were smokers (S) with normal lung function. All subjects gave written informed consent. This research was approved by the local research ethics committee.

2.2. Alveolar macrophage isolation

Alveolar macrophages were isolated from the lungs as previously described (Armstrong et al., 2009). Briefly, resected tissue was perfused with 0.1 M NaCl and the retrieved fluid was centrifuged (10 min, 400 *g*, room temperature). The cell pellet was re-suspended in RPMI-1640 media (Sigma-Aldrich, Poole, UK), layered over Ficoll-Paque (GE Healthcare, Buckinghamshire, UK), and centrifuged. Alveolar macrophage number and total cell number were assessed by trypan blue exclusion. Alveolar macrophages were re-suspended at a density of 1×10^6 per ml in RPMI-1640 media supplemented with 10% v/v foetal calf serum (Invitrogen, Paisley, UK), 2 mM L-glutamine (Invitrogen), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma-Aldrich). The average number of non-adherent cells was 1.5×10^6 per ml.

2.3. Alveolar macrophage culture

Freshly isolated macrophages were seeded onto flat bottomed 96-well plates at a concentration of 1×10^5 macrophages per well. There were two different culture conditions (Fig. 1); condition 1: macrophages were rested for 1 h in a 5% CO_2 humidified atmosphere at 37 °C before non-adherent cells were removed and fresh medium was added (short incubation protocol). Condition 2: macrophages were rested for 16 h in a 5% CO_2 humidified atmosphere at 37 °C and the following day non-adherent cells were removed and fresh medium was added (long incubation protocol).

After the addition of fresh media, macrophages cultured under both conditions were cultured for a further 24 h with and without LPS (1 μg/ml *Escherichia coli*, O26:B6, Sigma-Aldrich). Cell culture supernatants were removed and analysed for TNF-α and CXCL8 by ELISA according to the manufacturer's instructions (R&D Systems, Abbingdon, UK).

2.3.1. The effect of dexamethasone on LPS stimulated cytokine release

After the addition of fresh media, macrophages cultured under both conditions (short and long incubation protocols) were treated with dexamethasone (0.1–1000 nM) or vehicle control (DMSO 0.005%, now referred to as "vehicle") (both Sigma-Aldrich) for 1 h followed by LPS (1 μg/ml) stimulation for 24 h. Cell culture supernatants were removed and analysed for TNF-α and CXCL8 by ELISA.

2.3.2. The effects of dexamethasone and BIRB-796 on unstimulated cytokine release

After the addition of fresh media, macrophages cultured under both conditions were treated with either dexamethasone (1 μM), the p38 MAPK inhibitor BIRB-796 (1 μM, Sigma-Aldrich), or vehicle for 24 h (this timepoint was chosen to match the cell culture with LPS). Cell culture supernatants were removed and analysed for TNF-α, and CXCL8 by ELISA.

2.3.3. Phosporylation of p38 MAPK and NF-κB p65

Freshly isolated macrophages were seeded onto flat bottomed 6-well plates at a concentration of 1×10^6 macrophages per well for experiments investigating protein content by Western blot. Macrophages were cultured for 15 min, 30 min, 60 min (which represent the short incubation protocol and can be subdivided into 1a, 1b, and 1c respectively) and 16 h (condition 2), prior to non-adherent cell removal and the remaining cells were lysed. Samples were then prepared for Western blot.

2.4. Western blot

Cells were lysed in RIPA buffer [10 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Nonidet P-40, 0.25%]