Acute effect of cigarette smoke on TNF-α release by macrophages mediated through the erk1/2 pathway

Loutfig Demirjian, Raja T. Abboud, Hong Li, Vincent Duronio *

Department of Medicine, University of British Columbia and Vancouver Coastal Health Research Institute, Jack Bell Research Centre, 255-2660 Oak St, Vancouver, BC, Canada V6H 3Z6

Received 12 July 2005; received in revised form 6 March 2006; accepted 28 April 2006
Available online 4 May 2006

Abstract

Pulmonary emphysema is a major cause of mortality and morbidity in chronic obstructive pulmonary disease (COPD). Cigarette smoking is a major risk factor in the development of pulmonary emphysema. In this study, we investigated the acute effect of cigarette smoke in vitro on the production of tumour necrosis factor-α (TNF-α) using differentiated U937 cells, a macrophage model system. We found that stimulation of the macrophages with cigarette smoke media (CSM) leads to a rapid activation of extracellular-regulated kinases 1 and 2 (erk1/2), p90RSK and a transient decrease in phosphorylation of PKB/akt. The CSM also caused the subsequent induction of TNF-α release. Our studies revealed that U0126, an inhibitor of the erk1/2 pathway, markedly suppressed CSM-induced TNF-α release. Consistent with this finding, U0126 blocked CSM-stimulated erk1/2 phosphorylation, as well as phosphorylation of the downstream kinase, p90RSK. On the other hand, the PI3-K inhibitor, LY294002, and epidermal growth factor receptor (EGFR)-specific inhibitor, AG1478, did not suppress the release of TNF-α. Thus, CSM induction of TNF-α production by differentiated macrophages is regulated primarily via the erk1/2 pathway.

© 2006 Elsevier B.V. All rights reserved.

Keywords: Macrophage; In vitro cigarette smoke exposure; Tumour necrosis factor-α; erk1/2

1. Introduction

Currently, chronic obstructive pulmonary disease (COPD) is the fourth leading cause of death in the United States, and worldwide incidences are rising due to a widespread increase in tobacco consumption, particularly among women and adolescents [1]. COPD commonly refers to chronic bronchitis and emphysema. Pulmonary emphysema is defined as permanent airspace enlargement beyond the terminal bronchioles, with the destruction of alveolar walls and without obvious fibrosis [2]. Cigarette smoking is by far the major risk factor for development of pulmonary emphysema; however, other factors, such as pollution, α1-antitrypsin deficiency and occupational factors, also play a role.

A commonly accepted theory for the pathogenesis of cigarette smoke-induced emphysema is the protease–anti-protease hypothesis. Cigarette smoke induces an inflammatory response within the lung; these recruited inflammatory cells are composed of a heterogeneous population of neutrophils, macrophages and T lymphocytes (predominantly CD8+ cells). Macrophages and neutrophils are the two major inflammatory cells within the lung that release various proteolytic enzymes in excess of their natural inhibitors leading to tissue destruction and airway enlargement [3–5]. However, a key factor in the pathogenesis of emphysema is the inflammatory reaction within the lung; hence, identifying specific cytokines or mediators that orchestrate inflammatory cell recruitment to the lung may lead to a better understanding of the pathogenesis of the disease.

Macrophages orchestrate the primary response to external stimuli when defending the body; they are the major defence cells in the lower airspace of the lung in healthy nonsmokers and appear to have an essential role in the pathogenesis of COPD by accounting for most known features of the disease [6]. Bronchoalveolar lavage (BAL) fluid from smokers
compared to nonsmokers show a five-fold increase in the number of inflammatory cells in the lung, of which 85–90% are alveolar macrophages. Macrophages are predominant cells in the respiratory bronchioles of smokers; studies have shown a correlation between alveolar macrophage numbers and the extent of lung destruction in emphysema [7,8]. Activated macrophages release various inflammatory proteins, such as cytokines, chemokines and proteolytic enzymes. TNF-α is one of the acute pro-inflammatory cytokines secreted by macrophages and plays a central role in inducing the recruitment of inflammatory cells to the lung in a variety of pulmonary diseases [9].

TNF-α plays an important role in defence of the body against foreign pathogens such as virus, bacteria and fungi. In humans, TNF-α is synthesized as a 31-kDa precursor molecule; however, prior to secretion, the N-terminal sequence is removed leading to the release of a 17-kDa polypeptide [10]. TNF-α can bind to two different cell surface receptors p55 (TNFR1) or p75 (TNFR2). The main receptor in the TNF-α response is the p55 receptor which induces a variety of biological effects, whereas the p75 receptor is the prototype member of the death receptor family and is responsible for cytotoxic effects [11].

A variety of cells produce TNF-α, including neutrophils, T cells, macrophages, monocytes, epithelial cells, fibroblasts and smooth muscle cells [12,13]; however, the principle source of TNF-α in the lung is the alveolar macrophages. TNF-α production within the lung can play a central role in inducing the expression of adhesion molecules, including ICAM-1, VCAM-1 and E-selectin. It can also stimulate the expression of various cytokines such as interleukin(IL)-1, IL-6, IL-8, platelet-derived growth factor (PDGF), granulocyte macrophage colony stimulating factor (GM-CSF), monocyte chemoattractant protein-1 (MCP-1) and macrophage inflammatory protein 2 (MIP-2) [14–17]. This increase in the levels of chemokines, cytokines and adhesion molecules results in the activation and recruitment of neutrophils and macrophages to the lung and ultimately leads to tissue destruction.

There have been relatively few previous studies investigating effects of cigarette smoke products on cell signaling pathways in macrophages [18–20]. In this study, we investigated the effect of CSM on TNF-α release by macrophages and the relative importance of several signaling pathways in mediating TNF-α production due to CSM exposure. Use of CSM remains a good practical model to study changes in cellular responses induced by cigarette smoke. While some studies have focused on the effects of individual cigarette smoke components, our interest was in testing the effect of whole cigarette smoke, and therefore, CSM was used to mimic the in vivo soluble cigarette smoke components that are present during cigarette smoking.

2. Materials and methods

2.1. Materials

The human monocytic cells, U937 cell line was obtained from the American Type Culture Collection (Manassas, USA). RPMI-1640, l-glutamine, streptomycin–penicillin and fetal bovine serum were purchased from Stem Cell Technologies (Vancouver, BC, Canada). The PathScan Multiplex western cocktail 1 consists of phospho-p90RSK (Ser-380), phospho-PKB (Ser-473), phospho-erf1/2 (Thr-202/Tyr-204), phospho-S6 ribosomal protein (Ser 235/236), eIF4E antibody; the cocktail and MEK 1/2 inhibitor U0126 were purchased from Cell Signaling Technology (Beverly, MA). Anti-rabbit IgG horseradish peroxidase-conjugated secondary antibody, EGF-specific inhibitor AG1478 and PI3-K inhibitor LY294002 were purchased from Calbiochem (La Jolla, CA). The high molecular weight protein standard for Western blotting was purchased from BioRad (Hercules, CA). ECL (enhanced chemiluminescence) reagent was purchased from Amersham (Buckinghamshire, UK), and the human TNF-α Elisa kit was purchased from R&D Systems (Minneapolis, MN).

2.2. Cell culture

U937 cells were cultured in 75-cm² tissue culture flasks with RPMI medium supplemented with 10% heat-inactivated fetal bovine serum, 2 mM l-glutamine, 10 mM HEPES and streptomycin–penicillin in a humidified atmosphere of 95% air and 5% CO₂ at 37 °C. Cells in the culture flasks were maintained at a density of between 1 × 10⁶ cells/ml and 2 × 10⁸ cells/ml. Cells were harvested, then plated in 6-well tissue culture plates (2 × 10⁶ cells/2 ml/well) and stimulated with 20 ng/ml of phorbol myristate acetate (PMA, Sigma) for 48 h resulting in adherent differentiated macrophages. Adherent cells were washed twice with RPMI-1640, followed by the addition of fresh RPMI-1640 medium in the absence of PMA for an additional 24 h. Differentiated U937 cells represent a viable model for macrophages and therefore were used instead of primary human macrophages due to their availability and reproducibility over primary human cells, which are more difficult to obtain, and in which we have found signaling responses to be too variable to do the type of study we have described here.

On the day of smoke exposure, cells were washed twice with RPMI followed by the addition of fresh RPMI and incubated for 2 h prior to smoke exposure. For the experiments with MEK1/2 inhibitor U0126, EGFR-specific inhibitor AG1478 and PI3-K inhibitor LY294002, cells were pre-incubated with 10 μM of U0126, 10 nM of AG1478 and 6 μM of LY294002 for 1 h prior to smoke exposure and compared to control cultures that were not treated with inhibitors.

2.3. Exposure of cultures to cigarette smoke medium

Cigarette smoke generated from two medium tar commercial filtered cigarettes (Rothmans Blue) was bubbled through 25 ml of RPMI-1640. Eight puffs of 30 ml of smoke from each cigarette were withdrawn using a 60-ml syringe over 10 s and then bubbled through 25 ml of RPMI in 30 s. We chose 8 puffs of 30 ml of smoke from each cigarette by considering that a smoker will inhale about 30 ml of smoke per puff and that a cigarette will be consumed by a smoker with 8 puffs. The media pH was adjusted to 7.2–7.4 and diluted to achieve an optical density reading of 1.0 at 320 nm [35]. This was defined as 100% CSM. CSM was prepared in a non-sterile environment and thus to remove particles and to minimize risk of bacterial contamination, the CSM was filtered through a 0.2-μm pore filter. Prior to each smoke exposure, CSM was freshly prepared within an hour and diluted with medium to achieve the desired % concentration of CSM. Cells were treated with 0 (Control; air bubbled medium), 2.5, 5, 10, 15 and 20% of CSM, and cell viabilities following treatment were always greater than 95% as measured by trypan blue exclusion. The media were collected at 18, 24 and 48 h, centrifuged at 1000 rpm for 10 min and then stored at −70 °C.

2.4. TNF-α enzyme linked immunosorbent assay (ELISA)

TNF-α levels in the cell culture supernatant of control and CSM-exposed differentiated U937 cells were quantified by ELISA following the manufacturer’s instructions (R&D Systems). Briefly, 96-well plates (NUNC, MaxiSorp™ Surface) were coated with monoclonal anti-human TNF-α antibody (4 μg/ml) at 4 °C overnight. The wells were then washed 3 times with PBS–0.05% Tween 20 and then blocked with 1%BSA in PBS for 1 h. The plate was washed again, followed by the addition of samples and standards in duplicate, and incubated for 2 h at room temperature. The washing step was repeated and biotinylated goat anti-human TNF-α (75 ng/ml) was added and incubated for 2 h at room temperature. The wells were washed again, and streptavidin–HRP was added
and incubated for 30 min at room temperature. After a final wash, TMB (3, 5, 3′, 5′-tetramethylbenzidine) was added as a substrate and incubated for 1 h at room temperature, the reaction was stopped by the addition of H₂SO₄ (2N) and read at 450 nm.

2.5. Immunoblot analysis

Immunoblot analyses for phospho-proteins were performed on control and CSM-exposed differentiated U937 cells. The cells were serum-deprived for 2 h prior to stimulation with CSM. Control and treated cells (2 × 10⁶ cells) were washed 3 times with ice-cold phosphate-buffered saline (PBS) followed by the addition of 125 μl of ice-cold lysis buffer (50 mM Tris buffer pH 7.4, 1% Triton X-100, 10% glycerol, 100 mM NaCl, 2.5 mM EDTA, 10 mM NaF, 0.2 mM Na orthovanadate, 1 mM Na molybdate, 40 μg/ml PMSF, 1 μM pepstatin, 0.5 μg/ml leupeptin, 10 μg/ml soybean trypsin inhibitor). Lysates were collected and centrifuged at 13,000 rpm for 10 min at 4 °C, the supernatant containing protein was collected and the precipitates discarded. The protein concentrations of extracts were quantified by the Pierce protein assay. 5× sample buffer was added to each sample and heated at 90 °C for 4 min. High molecular weight standard (BioRad) and samples of equal protein quantities were loaded and separated on a 12% SDS-polyacrylamide gel.

The proteins were then transferred onto nitrocellulose paper by semi-dry blotting. After transfer, blots were stained with Ponceau Red for 1 min to confirm equal protein transfer to each blot. The membrane was then blocked with 5% skim milk in Tris-buffered saline containing 0.05% Tween 20 (TBST) for 1.5 h followed by incubation with the primary antibody overnight in 5% BSA/TBST at 4 °C. Membranes were then washed twice in TBST for 15 min at room temperature, then incubated with horseradish peroxidase-conjugated secondary antibody (1:2000 dilution) for 1 h at room temperature. The immunoblots were visualized with ECL reagent. Densitometry analysis of phospho-erk1/2 was performed using a Fluorchem TM 800 (Alpha Innotech Corporation, San Leandro, CA) high sensitivity digital camera system. Pixel density for the appropriate bands was captured, and the camera system software was used for the analysis of data.

2.6. Statistics

The data for TNF-α release are expressed as mean±standard deviation. Results were analyzed by the Student’s t test at a confidence level of P ≤ 0.05.

3. Results

3.1. Cigarette smoke media stimulates TNF-α release by macrophages

To evaluate the time effect of CSM on TNF-α release, macrophages were treated with 10% CSM for 3, 6, 9, 18, 24 and 48 h. Controls were macrophages treated with air-exposed media. TNF-α released in the media of the macrophages was assayed by ELISA. We observed that the effect of CSM on TNF-α release was time dependent. CSM induced TNF-α release as early as 3 h and peaked at 24 and 48 h (Fig. 1A). We then tested the effect of CSM on TNF-α release in a dose response experiment. Macrophages were treated with 0, 2.5, 5, 10, 15 and 20% CSM for 18, 24 and 48 h. Results indicate that CSM induces statistically significant release of TNF-α in a dose-dependent manner (Fig. 1B) following exposures to more than 2.5% CSM.

3.2. Activation of erk1/2 MAP kinases and deactivation of PKB by CSM

We investigated the effect of CSM on the activation of p90RSK, erk1/2, PKB and p70 S6 kinase in macrophages using a cocktail of specific antibodies. This was accomplished by immunoblotting with antibodies that detect only the phosphorylated and activated forms of the p90RSK, erk1/2 and PKB kinases, as well as an antibody detecting phosphorylated S6 ribosomal protein, a target of p70 S6 kinase. Macrophages treated with 5 and 10% of CSM showed the most significant change in the activity of erk1/2. There was a greater than twofold increase in the activity of erk1/2, with a corresponding consistent decrease in the activity of PKB after 15 min of CSM exposure; however, at 30 min of exposure, the activity of PKB was back to control levels (Fig. 2). The phosphorylation of p90RSK and S6 ribosomal protein was also assayed with antibodies to the phosphorylated forms of these proteins. Activation of the p90RSK kinase is at least partially dependent upon erk1/2 activation, while phosphorylation of the S6 ribosomal protein is a reliable measure of the activation of p70S6K. Exposure to CSM was found to activate p90RSK, while there was no consistent change in the activity of p70S6K, based on the level of phosphorylation of the S6 protein (Fig. 2).

3.3. Effect of MEK1/2 inhibitor U0126 on TNF-α release by CSM

To elucidate the intracellular signaling mechanism by which CSM induces TNF-α release, we investigated whether the activation of the erk1/2 pathway was necessary for CSM
induced TNF-α release by differentiated macrophages in vitro. Cells incubated with and without pretreatment with MEK1/2 inhibitor U0126 (10 μM) (Cell Signaling Technology, Beverly, MA) for 1 h were exposed to 2.5, 5, 10, 15 and 20% of CSM for 24 h. The inhibitor U0126 completely blocked TNF-α release, indicating that TNF-α release in response to CSM exposure is dependent on the activation of erk1/2 (Fig. 3). In contrast, PI3-K inhibitor LY294002 and EGFR-specific inhibitor AG1478 had no effect on CSM-induced TNF-α release by macrophages (Fig. 4). U0126, AG1478 and LY294002 did not affect the viability of the macrophages as measured by the trypan blue exclusion test.

4. Discussion

TNF-α production has been studied within humans by comparing groups of smokers, nonsmokers, smokers with COPD and healthy smokers without COPD, using samples from BAL fluid, induced sputum and serum. Kushner and coworkers report that the concentration of TNF-α in the pulmonary microenvironment is greater in healthy smokers compared to healthy nonsmokers [21]. However, in a study using induced sputum sample, a non-invasive method of studying airway secretion, showed that the concentration of TNF-α is greater in smokers with COPD compared to healthy smokers and nonsmokers [22]. Takabatake and coworkers found increased serum concentration of TNF-α in patients with COPD compared to healthy nonsmoking subjects [23]. The acute effect of smoke exposure on mice was studied by Churg and coworkers. A single acute smoke exposure from four cigarettes caused an increase in the gene expression of TNF-α from whole lung extract 2 h after smoke treatment [24].

There have been several studies on the effect of cigarette smoke on TNF-α production. Wang and coworkers showed that nicotine, a component of cigarettes, induces the release of TNF-α by cultured Ana-1 macrophages; furthermore, the TNF-α released induces the expression of adhesion molecules such as ICAM-1, VCAM-1 and E-selectin on endothelial cells which lead to an increase in adherence of monocytes to the endothelial cells [25]. In another study, alveolar macrophages from cigarette smokers were shown to release greater amounts of TNF-α at baseline and after treatment with LPS and IL-1β, compared to alveolar macrophages from nonsmokers [26]. However, there are other studies using blood monocytes, cultured alveolar macrophages and BAL fluid which state that cigarette smoke exposure reduces or has no effect on TNF-α production [27–31]. Our results concur with the previous studies showing that cigarette smoke does induce TNF-α release by macrophages, since we could easily detect increased TNF-α production in response to CSM exposure in both a time and dose-dependent fashion. We further explored the mechanism by which cigarette

![Fig. 2. CSM induces activation of erk1/2 and p90RSK, and decreased activation of PKB. [A] Phosphorylation of erk1/2, PKB, p90RSK and S6 ribosomal protein was analyzed by Western blot. 2 × 10⁶ macrophages/well were treated with 5 and 10% CSM for 15 and 30 min. Whole cell lysates were analyzed. Immunoblotting for eIF4E was included as a control for protein loading. [B] Quantitative densitometry result of phospho-ERK1/2 in (A) was normalized against eIF4E protein content.](image)

![Fig. 3. erk1/2 is a mediator in CSM-induced TNF-α release. [A] TNF-α release without (red bar) or with (blue bar) pretreatment with MEK 1/2 inhibitor U0126 (10 μM). The inhibitor blocked release of TNF-α following exposure to 2.5, 5, 10, 15 and 20% CSM for 24 h, indicating that release is dependent on ERK1/2 activation. Each bar represents the mean value ± S.D. from 3 independent experiments. TNF-α released in media was assayed by ELISA. (B) Western blot analysis from macrophages treated with U0126 for 1 h prior to treatment with 2.5, 5 and 10% CSM for 15 min. Treatment with U0126 did not affect the viability of U937 cells.](image)
Figure 4. Effect of AG1478 and LY294002 on TNF-α release. TNF-α release without (red bar) or with (blue bar) pretreatment with [A] PI3-K inhibitor U0126 (6 μM), [B] and EGFR-specific inhibitor AG1478. Both inhibitors had no effect on CSM-induced TNF-α release. Each bar represents the mean value±S.D. from 3 independent experiments.

In this study, we showed that cigarette smoke activates human macrophages and induces the release of TNF-α, and this release was dependent primarily on the activation of the erk1/2 signaling pathway. Activation of erk1/2 in turn induces the activation of the downstream kinase p90RSK and may have some effect on the activation of p70S6 kinase. We could show that inhibition of MEK1/2, the upstream activators of erk1/2, completely blocked CSM-induced erk1/2 phosphorylation, as well as p90RSK phosphorylation. However, there was no effect on phosphorylation of S6 ribosomal protein, and thus no effect on p70S6 kinase activity. Macrophages exposed to CSM showed a consistent decrease in the level of PKB phosphorylation, particularly at early times of exposure. Inhibition of the PI 3-kinase enzymes with LY294002, which inhibits activation of PKB, had no effect on the release of TNF-α in response to CSM. Also EGFR-specific inhibitor AG1478 did not effect CSM-induced TNF-α release by macrophages.

In conclusion, the current study demonstrates that macrophages exposed to cigarette smoke products are activated and release TNF-α. Since this single event has been shown to play a vital role in inflammatory responses, it represents a possible mechanism whereby cigarette smoke may lead to pulmonary conditions. Our studies also emphasize the importance of the erk1/2 pathway, which is activated by exposure to CSM and which is necessary for the CSM-induced release of TNF-α. Further studies will be required to fully understand how components of cigarette smoke activate signaling pathways and to investigate other pathways that may also be playing a role in macrophage activation. Furthermore, it will be important to test these same pathways and the response to CSM in primary human macrophages.

Acknowledgments

Supported in part by grants from the British Columbia Lung Association and the Canadian Institutes of Health Research. We would also like to thank Rosa Garcia, Johnny Chen and Payman Hojabrpour for their technical assistance and helpful suggestions.

References


