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Intracellular and Extracellular Cytokines in A549 Cells and THP1 Cells Exposed to Cigarette Smoke

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Abstract

Cigarette smoke (CS) activates inflammatory cells and increases cytokine levels producing local and systemic inflammation. To assess changes in intracellular and extracellular cytokine levels we used human epithelial (A549 cells) and monocyte (THP-1) cell lines grown for 24 h in cigarette smoke-conditioned media. Cytokines were assessed using immunostaining/flow cytometry and ELISA assay. In THP1cells, grown in CS-conditioned media, the intracellular interleukins IL-1β, IL-6, and IL-10 increased by more than tenfold, while less significant increases were found in A549 cells. IL-1α and IL-1β, but not IL-6 or IL-10, were increased in the culture media, while IL-2 was raised by about fivefold only in the culture medium of A549 cells. IL-4, IL-6, IL-8, IL-10, IL-12, and tumor necrosis factor alpha were undetectable, while only a slight increase was observed in extracellular IL-17A (by about 60 %) in the medium of A549 cells and by about 115 % in the medium of THP1 cells. The interferon gamma (IFNγ) was increased by about eightfold, but only in the medium of THP1 cells grown with CS. We conclude that IL-1 and INFγ are the key cytokines responsible for pro-inflammatory signaling in epithelial cells and monocytes, respectively, exposed to cigarette smoke.

Keywords

A549 cells • Cell culture • Cigarette smoke • Cytokine • Inflammation • THP1 cells

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1 Introduction

Cigarette smoke (CS) is one of the leading causes of death and an important risk factor for systemic and respiratory tract diseases. Chronic CS exposure causes structural and functional changes in

the respiratory tract but detailed mechanisms remain obscure. Long-lasting smoking may generate different pathologies including chronic obstructive pulmonary disease (COPD), epithelial cell tumors, cardiovascular disease, but also an increased incidence of asthma and respiratory infections (Chang et al. 2015). Respiratory epithelium is the main target of highly toxic, fresh CS. It has been shown that epithelial integrity and immunity is significantly affected by smoke exposure (Crotty Alexander et al. 2015). CS is clearly detrimental to lung epithelium and it mobilizes and activates alveolar macrophages producing pro-inflammatory mediators, reactive oxygen species, and proteolytic enzymes (Sarma and Ward 2011). On the other hand, several chemicals of CS have both anti-inflammatory and immunosuppressive proprieties (Das et al. 2012; Kalra et al. 2000). Due to the complex nature of CS, diversity of smoking habits, and different experimental models of CS exposure, published data are inconsistent. It has been shown that smoking may suppresses cytokine expression in asthma, but at the same time it evidently worsens lung functions (Tamimi et al. 2012). It seems credible that at early stages circulating monocytes adhere to damaged epithelial cells repeatedly exposed to extremely toxic constituents of CS and migrate into respiratory tissue contributing to morphological and functional changes. We have previously shown that lung epithelial cell line (A549 cells) grown for 24 h in a CS-conditioned culture medium die due to chemical and oxidative stress while human monocyte cells (THP1 cell line) grown in similar CS-saturated medium become activated (Holownia et al. 2015). Moreover, CS toxicity to A549 cells is significantly lower when the cells were co-cultured with THP1 cells sharing a common culture medium. The purpose of the present study was to further explore the same model and investigate the effects of CS on immune response, particularly on intracellular cytokine levels and on cytokine secretion to the culture medium.

2 Methods

2.1 Cell Culture

A549 (ATCC® CCL185™) cells grown in ATCC-formulated F12K medium supplemented with 10 % fetal bovine serum (FBS) and THP1 cells (ATCC® TIB202™) grown in ATCC-formulated RPMI 1640 medium, supplemented with 2-mercaptoethanol to a final concentration of 0.05 mM and with FBS to a final concentration of 10 % were used in this study. Cells were maintained in 37 °C in an incubator in a humidified atmosphere containing 5 % CO_2. For particular experiments cells were plated out onto 6 well plates and were grown in control or smoke conditioned media for 24 h.

2.2 Preparation of CS-Conditioned Media and Cell Treatment

CS-conditioned medium was prepared using full-strength Red Marlboro cigarettes (Phillip Morris; Cracow, Poland) containing 8 mg of tar, 0.6 mg of nicotine, and 9 mg of carbon monoxide per cigarette. To prepare smoke-conditioned media cigarette filters were removed and smoke was passed through culture media (4 cigarettes/100 ml of medium) using low pressure vacuum pump. Freshly prepared SC media were diluted with standard media to obtain 30 μM nitrate/nitrite (colorimetric reaction with Griess reagent) content in each batch. CS-conditioned media were subsequently filtered using 0.22-μm filters and were applied immediately to cell culture. Cells were grown in CS-conditioned media for 24 h and then were tested for intracellular interleukin content. Additionally, levels of interleukins IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17A, interferon gamma (IFNγ), tumor necrosis factor alpha (TNFα), and granulocyte-macrophage colony-stimulating factor (GM-CSF) were determined in the culture media to quantify cytokine release and leaking.

2.3 Intracellular Interleukin IL-1ß, IL-6, and IL-10

Intracellular interleukins were quantified in Triton X-100 permeabilized A549 or THP1 cells using interleukin-specific primary antibodies, corresponding fluorescent secondary antibodies, and flow cytometry detection. Briefly, cells were washed twice with PBS and counted. 10^6 cells were subsequently permeabilized with Triton® X-100 (1 % in PBS) and rabbit polyclonal antibodies to IL-1β, IL-6, or IL-10 (all from Abcam; Cambridge, UK) were separately added to each sample. After 10 min of incubation at room temperature a secondary, isotype specific fluorescein-bound antibody (Sigma-Aldrich, Poznan, Poland) was added and samples were run on an Epics XL flow cytometer (Coulter Electronics, High Wycombe, UK). Reference samples were prepared using the same isotype, but with unspecific primary antibodies (Abcam; Cambridge, UK). Two thousand total events were collected per sample.

2.4 Extracellular Cytokine Profiles

To quantify cytokines released from the cells to their culture media, the Multi-Analyte Inflammatory Cytokine ELISArray Kits (Qiagen, Manchester, UK) were used. A panel of 12 pro – and anti-inflammatory cytokines including IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17A, IFNγ, TNFα, and GM-CSF were simultaneously analyzed using a sandwich-based enzyme-linked immunosorbent assay (ELISA) in a microplate, coated with cytokine-specific antibodies. After washing, biotinylated antibodies, avidin-horseradish peroxidase conjugate, and peroxidase substrate was added to produce blue color. Samples were compared with the corresponding positive controls (extracellular cytokines) and with matching data from the CS-conditioned media. Microplate reader (KC junior, BioTek Instruments; Highland Park, VT) was used to read the absorbances at 450/570 nm according to the manufacturer's protocol.

2.5 Statistical Analysis

Statistical analysis was performed with a statistics package-Statistica 6.0 software (Statsoft; Cracow, Poland) using one-way or two-way ANOVA followed by the Bonferroni *post hoc* test for selected pairs of data. Results were expressed as means of 4–6 assays ±SD. A p-value of less than 0.05 was considered statistically significant.

3 Results

Table 1 shows intracellular IL-1β, IL-6, and IL-10 levels (flow cytometry), and pro– and anti-inflammatory cytokines IL-1α, IL-1β, IL-2, IL-4, IL-5, IL-6, IL-8, IL-10, IL-12, IL-13, IL-17A, and GM-CSF profiles in culture media of control A549 and THP1 cells and in culture media of A549 and THP1 cells grown for 24 h in the CS-conditioned medium. In both cell types grown for 24 h in the CS-conditioned medium, intracellular interleukin IL-1β, IL-6 (Fig. 1), and IL-10 were significantly increased. Greater increases in intracellular interleukins were observed in THP1 cells, where IL-1β, IL-6, and IL-10 increased by about 11-fold ($p < 0.01$), 13-fold ($p < 0.01$), and 14-fold ($p < 0.01$), respectively. The corresponding values in A549 cells were about threefold ($p < 0.01$), fivefold, and sevenfold ($p < 0.01$). The highest observed increase was detected in the pleiotropic interleukin - IL-10 in THP1 exposed to CS.

Considering changes in the cytokine profiles in the culture medium, more than half of 12 the cytokines tested were undetectable. Significantly increased levels were found in IL-1α (by more than 2 times, $p < 0.01$) in the culture medium of A549 cells and by more than 4 times ($p < 0.01$) in the culture medium of THP1 cells. Similar, but more pronounced, changes were observed in IL-1ß. Its level increased by more than 3 times ($p < 0.01$) and by more than twice ($p < 0.01$) for A549 and THP1 cells, respectively. IL-2 was increased by about fivefold ($p < 0.01$), but only in the culture medium of A549 cells and was not

Table 1 The effect of cigarette smoke (CS) on intracellular interleukin 1β (IL-1β), interleukin 6 (IL-6) and interleukin 10 (IL-10) and on pro- and anti-inflammatory cytokine profiles (IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12, IL-17A, IFNγ, TNFα and GM-CSF) in culture media of A549 cells and THP1 cells grown for 24 h in CS-conditioned medium

Intracellular cytokines (relative units)				
	A549 cells		**THP1 cells**	
	Control	**CS**	**Control**	**CS**
IL-1β	100 ± 27	330 ± 48**	100 ± 21	1124 ± 311**
IL-6	100 ± 22	533 ± 67**	100 ± 33	1321 ± 433**
IL-10	100 ± 19	664 ± 78**	100 ± 31	1420 ± 632**
Cytokine levels in culture media (relative units)				
IL-1α	100 ± 27	223 ± 29**	100 ± 29	433 ± 77**
IL-1β	100 ± 29	330 ± 42**	100 ± 29	237 ± 65**
IL-2	100 ± 19	520 ± 66**	ND	ND
IL-4	ND	ND	ND	ND
IL-6	ND	ND	ND	ND
IL-8	ND	ND	ND	ND
IL-10	ND	ND	ND	ND
IL-12	ND	ND	ND	ND
IL-17A	100 ± 33	163 ± 29*	100 ± 43	215 ± 41*
IFNγ,	ND	ND	100 ± 41	855 ± 81
TNFα	ND	ND	ND	ND
GM-CSF	100 ± 22	74 ± 41	100 ± 28	143 ± 34

Cytokine levels in control cells are expressed as 100 relative units

IL interleukin, *IFNγ* interferon gamma, *TNFα* tumor necrosis factor alpha, *GM-CSF* granulocyte-macrophage colony-stimulating factor

ND not detectable

*p < 0.05; **p < 0.01 for comparisons with the corresponding control cells

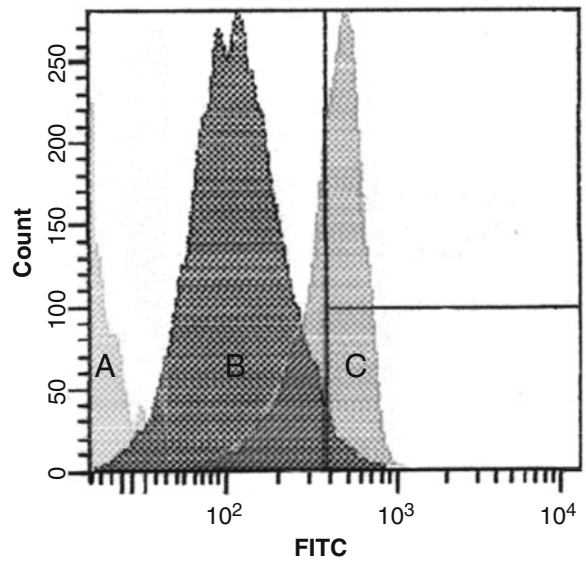

Fig. 1 Histograms of flow cytometry fluorescence of intracellular interleukin 6 (IL-6) in control A549 cells (*B*) and A549 cells grown for 24 h in cigarette smoke-conditioned medium (*C*). Cells were permeabilized, incubated with primary antibody specific to IL-6 and then with fluorescein isothiocyanate (FITC), secondary antibody. Cells were run on epics XL flow cytometer, control gating was set using naïve A549 cells, unspecific primary antibody, and the fluorescent secondary antibody (isotype control; *A*)

detected in THP1 cells. IL-4, IL-6, IL-8, IL-10, and IL-12 were undetectable, while only a slight increase was observed in IL-17A (by about 60 %; $p < 0.05$) in the culture medium of A549 cells and by about 115 % ($p < 0.01$) in the culture medium of THP1 cells. TNFα was not identified, while IFNγ was notably increased (by about eightfold; $p < 0.01$) when compared to control values, but only in the media of THP1 cells grown for 24 h with CS. GM-CSF levels were detectable, but were insignificantly affected by CS exposure.

IL-1β was the only cytokine in A549 and THP1 cells that increased both intracellularly and extracellularly after smoke exposure. Highly elevated levels of intracellular IL-6 and IL-10 upon exposure of A549 and THP1 cells to CS did not result in increased IL-6 and IL-10 levels outside the cells.

4 Discussion

Chronic CS exposure causes structural and functional changes in the respiratory tract, but detailed mechanisms remain elusive. It seems that the primary event in CS cytotoxicity involves epithelial cells, which further activate immune cells to produce inflammation and a variety of time-dependent, morphological and functional alterations. In this study we characterized the epithelial cell interactions with macrophages in a cell culture-based model consisting of human epithelial – A549 cell and monocyte – THP1 cell lines. In our previous study we have shown that the A549 cells and THP1 cells have a contrasting response to CS. The nature and intensity of oxidative and chemical stress induced by CS in the A549 and THP1 cells were different, as CS was significantly more toxic to the A549 cells. Moreover, when both cell types were grown in co-culture sharing a common culture medium, both naïve and CS-pretreated THP1 cells protected the A549 cells against CS toxicity, but also more prone to die (Holownia et al. 2015). In the present report we assessed major cytokine levels inside and outside the A549 and THP1 cells grown in a monoculture, and exposed to CS. We demonstrate that in the A549 and THP1 cells grown for 24 h in the CS-conditioned medium, IL-1β, IL-6 and IL-10 were significantly elevated. Particularly high increases were found in the THP1 cells, where IL-1β and IL-6, and anti-inflammatory IL-10 were increased more than 10 times. In spite of that, both IL-6 and IL-10 were not detected in the culture media, while the pro-inflammatory IL-1β was significantly increased both inside and outside the A549 and THP1 cells.

Inflammatory cytokines play a critical role in coordinating the inflammatory response and are increasingly important targets for therapeutic interventions. Experimental and clinical data show that CS can activate inflammatory cells and stimulate release of inflammatory cytokines (Crotty Alexander et al. 2015). CS increases the levels of IL-1β, IL-6, IL-8, TNFα, and GM-CSF (Zuo et al. 2014; Arnson et al. 2010). In smokers, mononuclear cells produce increased amounts of pro-inflammatory IL-1β, IL-6, and TNFα, and exhibit enhanced response to mitogen-stimuli (Zeidel et al. 2002). Interleukins are classified as pro– and anti-inflammatory, based mostly on their effects on leukocytes. Anti-inflammatory interleukins include IL-4, IL-10, IL-11, and IL-13, while IL-1, IL-6, IL-8, and IFNγ are pro-inflammatory cytokines (Siebert et al. 2015). IL-1 is a potent pro-inflammatory cytokine, which may be activated by tissue damage. It is synthesized as inactive protein and then it is activated by caspase-1 and released outside the cells by several ways, including exocytosis, active transport, cell lysis, and others (Netea et al. 2010). It has been shown that IL-1β is increased in sputum and lavage fluid of smokers (Chung 2006; Ekberg-Jansson et al. 2001). Monocytes isolated from cigarette smokers also produce more IL-1β than do monocytes from non-smokers (Zeidel et al. 2002). Bronchial epithelial cells exposed to CS-conditioned medium also release increase the amount of IL-1β compared with controls (Rusznak et al. 2001). Our present results confirm earlier data and indicate that both macrophages and epithelial cells may increase IL-1β levels and enhance IL-1β export

in response to CS. We did not assess the intracellular IL-1α, but increased IL-1α levels were found in the culture media of both cell types exposed to CS. It is possible that IL-1 plays a critical role as inflammatory cytokine in CS exposure.

In the present study, CS significantly increased intracellular IL-10 in the THP1 and A549 cells, but IL-10 remained undetected in the culture media. IL-10 is a pleiotropic cytokine. It downregulates the expression of lymphocyte Th1-derived cytokines and may block pro-inflammatory signaling related to nuclear factor kappa beta (NF-κb) (Sprague and Khalil 2009). It has been found that nicotine may down-regulate IL-10 (Allam et al. 2013). Recently published data also describe the nicotine-induced impairment of IL-10 production by macrophages (Van Zyl-Smit et al. 2014). Our results demonstrate that cells may overexpress or accumulate IL-10 in response to CS smoke, but the protein is not released outside the cells. Further studies are required to explore a detailed causal association between increased cytokine level and its decreased release.

Another extracellular cytokine increased by CS was IL-17A, which is considered as a crucial interleukin that regulates lung immunity and inflammation. Activation of innate cellular sources of IL-17A may mediate the increase in macrophage infiltration of CS-exposed lungs (Bozinovski et al. 2015). Elevated IL-17A has also been observed in sputum collected from exacerbated COPD patients (Roos et al. 2015). Recent knockout studies indicate that IL-17A is not involved in CS-induced loss of lung functions, but rather contributes to normal lung homeostasis (Voss et al. 2015). In our experimental model, IL-17A was increased by about twofold, which is similar to recently published clinical data in COPD patients (Montalbano et al. 2015). In that study, expression of IL-17A in the epithelial cells of distal airways positively correlated with the total packs per year in COPD patients, which might influence the rate of apoptosis and proliferation.

In the present study, INFγ increased in THP1 cells, but not in A549 cells, exposed to CS. An increased level of INFγ in inflammatory cells has been described in several models of CS toxicity (Ahn and Aggarwal 2005), which can reflect the activation of inflammatory pathways. On the other hand, CS did not induce INFγ in our epithelia-derived cells, indicating different regulatory mechanisms.

In conclusion, the present findings indicate that damaged and chemically stimulated epithelial cells exposed to CS activate internal and external inflammatory response, cytokine secretion, and possibly stimulate inflammatory cells. IL-1 seems to play a major role in mediating the CS effects in epithelial cells, while the INFγ−related signaling may be important in macrophages.

Conflicts of Interest The authors had no conflicts of interest to declare in relation to this article.

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