INTRODUCTION

Cigarette smoke (CS) has been associated with a variety of human pathologies, including cardiovascular disease and cancer. Human monocytes are prevalent in oral and respiratory mucosa and may be affected by exposure to CS, which induces oxidative stress. As a result, up-regulation of nuclear factor-kB (NF-kB) may occur. Our aims were to analyze a possible regulatory effect of CS on NF-kB activity in human monocytes. Human monocyte cell lines were exposed to CS in vitro. Our findings show that in vitro exposure to CS did not affect viability of human monocytes and was associated with increased production and secretion of IL-8 and up-regulation of certain C-C chemokines. Inhibition of NF-kB with curcumin or parthenolide resulted in a decrease of IL-8 secretion. CS also impaired the differentiation of monocytes. However, induced secretion of IL-8 from differentiated monocytes was not impaired. Our results indicate that exposure to CS stimulates pro-inflammatory activity of human monocytes through the activation of NF-kB pathway and also interferes with monocyte differentiation, which could play a role in the carcinogenic effects of cigarette smoking.

**Key words:** cigarette smoke, chronic obstructive pulmonary disease, nuclear factor-kappaB, monocytes, IkBα, interleukin-8
They observed that the resulting inflammation was associated with activation of the transcription factor NF-κB. Despite a growing body of evidence describing the effects of CS in vitro and in vivo, the signaling pathways involved in smoke-induced responses are currently still the subject of intensive investigation.

In the present work we wished to elucidate the effect of CS on human monocytes. Specifically, we examined the hypothesis that CS exposure may affect inflammatory process in human monocytes and macrophages through NF-κB activation. Our results indicate that exposure to CS stimulates pro-inflammatory activity of human monocytes through the activation of NF-κB pathway and also interferes with monocyte differentiation, which could play a role in the carcinogenic effects of cigarette smoking.

MATERIAL AND METHODS

Cell lines

Pro-monocytic human cell line used in this study was obtained from American Type Culture Collection (ATCC, Manassas, USA). Cells were maintained in RPMI/FBS, 100 U/ml penicillin and 100 µg/ml streptomycin. Monocytes were then stimulated to differentiation-THP-1 cells were centrifuged (1200 rpm for 10 min), resuspended in differentiation medium (RPMI1640, supplemented with 7.5%/FBS and antibiotics) and plated at a density of 2x10⁶ cells per ml in culture plates (100 mm). 30 nM of phorbol myristate acetate (PMA) (Sigma-Aldrich, St. Louis, MO) was added to adherent macrophages for the induction of differentiation. Cells were cultured for 4 days and subsequently washed with RPMI 1640 medium to remove PMA and culture one more day. Cultures were kept at 37°C in a humidified incubator (5% CO₂, 95% air). The experiments with monocytes were performed immediately following the above preparation, unless noted otherwise. Cell viability was assessed at various time points by Trypan Blue staining, both in treated and control cells.

Pharmacological treatment of the cells

At the beginning of each experiment, culture medium was replaced by fresh medium in which various substances and reagents were dissolved. All chemicals were obtained from Sigma-Aldrich (St. Louis, MO) and prepared according to the manufacturer’s instructions. The following substances were used: (i) 30 nM of phorbol myristate acetate (PMA) (Sigma-Aldrich, St. Louis, MO) was added to adherent macrophages for the induction of differentiation. Cells were cultured for 4 days and subsequently washed with RPMI 1640 medium to remove PMA and culture one more day. Cultures were kept at 37°C in a humidified incubator (5% CO₂, 95% air). The experiments with monocytes were performed immediately following the above preparation, unless noted otherwise. Cell viability was assessed at various time points by Trypan Blue staining, both in treated and control cells.

CS exposure method

Cigarettes used in this study were commercial Time cigarettes containing 14 mg of tar and 0.9 mg of nicotine (Time Cigarettes, Dubek Ltd., Tel Aviv, Israel). Exposure to CS was carried out in a gas phase system previously described (2). Culture medium samples and cultured cells were removed from the bottles for biochemical analysis at time 0 and at 5, 10, 15, 20, and 60 min.

Flow cytometry

Preparation of THP1 cells for FACS analysis: after differentiation, adherent macrophages were detached from culture plates, washed and centrifuged at 1200 rpm for 10 min. After centrifugation cell were resuspended in PBS and transferred into FACS tubes. Each tube contains 0.5x10⁶ cells in 200 µl PBS. Cell staining and FACS analysis: cells were prepared as described above. For each tube 5 µl of antibody conjugated with FITC- or PE was added and incubated for 20 min. Stained cells were washed with PBS, centrifuged at 1200 rpm for 10 min and resuspended and fixed in 150 µl of PBS together with 50 µl of formaldehyde 37%. Data for 2-color analysis were collected on a FACSCalibur and analyzed using CellQuest software.

ELISA

The secretion of IL-8 chemokine in cell supernatants after different smoking intensities was examined by commercial ELISA kit, Human IL-8/NAP-1. According to manufacturer's direction, plates were read in an ELISA reader (Tecan SPECTRA Rainbow Thermo) at 450 nm. Total IL-8 concentrations in the supernatants were calculated using a reference standard curve of purified IL-8 standard.

Preparation of total cell lysates

Monocytes: after treatment, cells were centrifuged at 1200 rpm for 10 min. Supernatants were removed and kept for later analysis. For cytoplasmic extracts cells were resuspended in 300 µl of Commercialize Lysis buffer, and incubated for 15 min on ice.

Macrophages: after treatment, the culture plates were put on ice, the medium was collected for later analysis, and the cells washed with cold PBS (without Ca²⁺, Mg²⁺). For phosphorylation levels of IκB, 1 ml of lysis buffer were added to the plates, for total protein extraction, 1 ml of whole protein lysis buffer was added. The attached macrophages were collected by scratching with rubber policeman. The liquid containing the detached cells was transfer to tubs and incubated for 15 min on ice. In the end of lysis, both cases, cells were cold (4°C) centrifuged for 15 min at maximum speed. Supernatants of cell lysates containing cytoplasmic fraction or total cell proteins were removed to new tubes and kept at -20°C.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and western blot analysis

SDS-PAGE and western blot analysis were performed as described (18). 10 µg of protein samples were run on each lane. The equality of sample loading in each lane was confirmed by Coomassie Brilliant Blue gel staining and by Ponceau Red membrane staining following the blotting. Primary antibodies used were: anti-phospho-IκBα (Ser32/36), mouse, monoclonal (Cell Signaling, Danvers, MA), anti actin, goat, polyclonal (Santa Cruz, CA). Secondary antibodies were sheep anti-mouse IgG HRP-conjugated (Amersham, France), donkey anti-goat IgG HRP-conjugated (Santa Cruz, CA).

Human cytokine antibody array

The detection of cytokine profile was using Human Cytokine Antibody Array commercial kit according to manufacturer's directions. This kit is based on the protein expression array. The membrane which was used was array 5, containing antibodies for 79 different cytokines, together with positive and negative controls. The secretion of IL-8 in cell supernatants was examined after smoking for 60 min (intensity 100 mmHg) and after no smoking at all. Samples of supernatants were incubated with the membrane for 2 h. After that biotin-conjugated anti-cytokines cocktail was added, followed by HRP-conjugated streptavidin incubation. Visualization of bound antibodies was performed by detection buffers provide with the kit, based on ECL reaction. Developing was made in tan image master device (ImageMaster VDS-CL Amersham, France). The level of expression of the different cytokine was analyzed with TOTAL-LAB software.
Statistical analysis was performed using unpaired Student’s t-test. To determine statistical significance, the ranges, means and SD were computed. Results are reported as means±SD. Statistical significance was set at P<0.05.

RESULTS

As CS induces oxidative stress in cells, and as oxidative stress may activate NF-κB, we wanted to test whether CS will have a pro-inflammatory effect on human monocytes. For that purpose we screened, by cytokine array, the profiles of cytokines secreted by monocytic cells which were exposed to CS. As can be seen in Fig. 1, the main cytokine that is upregulated significantly in THP-1 cells as a result of CS exposure is the chemokine IL-8. The other upregulated chemokines and cytokines are MCP-1, MIP-1β GRO, and IL-10. No other cytokines or chemokines have been upregulated significantly. Pro-inflammatory cytokines like TNF-α and IL-6 were not expressed in THP-1 cell line, with or without exposure to CS. In order to elucidate whether CS had an effect on cell viability, we exposed monocytes (THP-1) to different intensities of CS for 1 hour and afterwards stained them with Trypan Blue. Exposure to CS did not affect significantly the viability of human monocytic cells (data not shown).

The demonstration of increased IL-8 secretion from monocytic cells exposed to CS raised the question of whether the increase is only a result of release of stored IL-8 or is there also de novo generation of IL-8. For this purpose, THP-1 cells were exposed to CS (100 mmHg) for different periods of time and then cultured overnight. IL-8 concentration was tested both in supernatants and in cell lysates. As shown in Fig. 2, initially, a stored amount of IL-8 in the cells is secreted following exposure to CS.

![Fig. 1. Cytokine profile in human monocytic cells exposed to CS. THP-1 cells were exposed to CS (100 mmHg) for 60 min and then cultured overnight. Cell free supernatants were collected and assayed by a cytokine array.](image1)

![Fig. 2. IL-8 concentration in supernatants and lysates of THP-1 cells exposed to CS. IL-8 concentration in both supernatants and cell lysates was determined by ELISA. Results of 2 experiments are shown as means±SD.](image2)

![Fig. 3. Effect of NF-κB inhibitors on IκB phosphorylation in THP-1 cells exposed to CS. THP-1 cells were exposed to CS (100 mmHg) for various time periods and cellular samples were taken for protein analysis. A-untreated cells and then exposed to CS; B - cells pretreated for 1 h with parthenolide (15 µM) and then exposed to CS; D - cells pretreated for 1 h with curcumin (25 µM) and then exposed to CS; C and E - actin blots are shown as controls.](image3)

![Fig. 4. Curcumin inhibits IL-8 secretion from THP-1 cells exposed to CS. THP-1 cells were pre-treated for 60 min with curcumin and then exposed to CS (100 mmHg) for 60 min and further cultured overnight. Cell free supernatants were collected and IL-8 concentration was determined by ELISA. Results of 3 experiments are shown as means±SD.](image4)
However, CS also stimulated de novo generation of fresh IL-8 in an amount which parallels to the secreted amount.

Next, we wished to check whether the increase in IL-8 secretion due to exposure of monocytic cells to CS is also associated with NF-κB activation. For that purpose we tested phosphorylation of IκBα with or without the presence of NF-κB inhibitors in association with CS exposure. As shown in Fig. 3A, IκBα becomes maximally phosphorylated after 10 min exposure to CS, which indicates loss of inhibition and further activation of NF-κB pathway. Pretreatment with parthenolide (Fig. 3B) or curcumin (Fig. 3D) abolished phosphorylation of IκBα. These results point to the role of CS in NF-κB activation.

As we have shown that CS stimulates IL-8 secretion and NF-κB activation, we wished to check whether inhibition of NF-κB pathway will inhibit IL-8 secretion. As shown in Fig. 4, curcumin (25 µM) inhibited the secretion of IL-8 by almost 70%. Similar results were also obtained with parthenolide (Fig. 5).

In contrast, differentiated THP-1 cells (THP1-MΦ) were not affected significantly by CS exposure (data not shown). Thus, we wished to test whether in the pre-differentiation stage, exposure of human monocytes to CS will have any effect on IL-8 secretion from THP1-MΦ. However, pre-differentiation CS exposure of THP-1 cells did not affect IL-8 secretion from THP1-MΦ (data not shown). Faced by the different effects of CS exposure on THP1-MΦ, we wished to check whether inhibition of NF-κB pathway will inhibit IL-8 secretion. As shown in Fig. 4, curcumin (25 µM) inhibited the secretion of IL-8 by almost 70%. Similar results were also obtained with parthenolide (Fig. 5).

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on IL-8 secretion from immature or differentiated monocytic cells, we wished to test whether CS would interfere with the differentiation process of human monocytes. THP1-MΦ were exposed to CS for up to 60 min and then cultured overnight.

We can see from Fig. 6 that CS is affecting the morphology of THP1-MΦ. Macrophages lose their "differentiated morphology" and become more round and less elongated and sticky, and look less viable. As we noted an effect of CS on differentiated mononcytic cells, we wished to check whether CS may interfere with the differentiation process. We exposed THP-1 cells to CS for different periods of time and then incubated them with PMA for 5 days. Differentiated mononcytic cells (THP1-MΦ) were stained for CD4 and CD14 and analyzed by FACS for the relevant expression. As shown in Fig. 7, exposure to CS impaired CD4 and CD14 expression on THP1-MΦ cells. The maximum effect was achieved with 100 mmHg partial pressure and 60 min exposure and has been associated with a decrease of up to 68% in CD4/CD14 expression.

**DISCUSSION**

In the pulmonary system, CS is implicated in the development and exacerbation of chronic bronchitis, asthma, COPD, and lung cancer (2,3). Tobacco use has been reported to be the main cause of 90% male and 79% female lung cancers. The risk of lung cancer development is 20-40 times higher in lifelong smokers compared to non-smokers (19). Studies examining central airways of smokers have shown that T lymphocytes and macrophages are the predominant cells infiltrating the airway wall, whereas neutrophils, which are scarce in the airway wall, are increased in the airway lumen (20,21). Oxidative stress induces IL-8 release from lung alveolar epithelial cells; however, the molecular mechanisms of this process have not been thoroughly investigated (22). CS contact with various human tissues, in particular human alveolar cells, is associated with nitric oxide (NO) and other ROS exposure. The resulting oxidative stress may affect the regulation of NF-κB and macrophages play a central role in conferring a first line defense against invading pathogens. Inflammatory mediators secreted by activated macrophages play a critical role in the protective function against exogenous stimuli. We found that differentiated monocytic cells (THP1-MΦ) secreted spontaneously high amounts of IL-8, and CS exposure did not affect it to any further extent (data not shown). Studies have shown that increased cigarette smoking intensity is associated with dose-dependent increases in BALF macrophage number and in the concentration of IL-8 in BALF (26). BALF macrophages in smokers are exposed repeatedly to CS, and this may affect IL-8 secretion.

**ROS and RNS**

ROS and RNS have several effects on bronchial airways, which may increase the inflammatory response. These effects may be mediated by direct actions of ROS/RNS in the airways, or indirectly via activation of signal transduction pathways and transcription factors like NF-κB (25). We assumed that activation of NF-κB is due to oxidative stress brought up by ROS/RNS originating from CS. Since CS consists of 1015-1017 free radicals per puff, it is therefore predictable that CS induces oxidative stress within cells. We found that CS exposure is associated with the phosphorylation of Ik-Bα (Fig. 3A), which indicates loss of inhibition and further activation of NF-κB pathway. In accordance, IL-8 secretion was increased (data not shown). Inhibition of NF-κB abolished the phosphorylation of Ik-Bα (Fig. 3B and D), further indicating that CS exposure was associated with NF-κB activation. Inhibition of NF-κB in monocytic cells was also associated with suppression of CS-induced secretion of IL-8 (Figs. 4-5). These results also suggest that CS-induced release of IL-8 is associated with activation of the NF-κB pathway. Laan et al. (5) have found that pretreatment of bronchial epithelial cells with CS is associated with decreased LPS-induced secretion of IL-8, mediated through suppression of activated protein 1(AP-1) activation. We did not test AP-1 activation in the current study, but the results clearly indicated that CS-induced NF-κB activation is associated with increased IL-8 secretion.

**NF-κB activation by ROS/RNS**

NF-κB activation by ROS/RNS is cell specific (23) and depends on the level and types of ROS/RNS. Thus, NF-κB activation does not seem to be a universal response to oxidative stress and the molecular mechanism of ROS/RNS-mediated NF-κB upregulation is complex and largely unknown. However, it seems that in our system, IL-8 release and de novo generation is associated with a classical pathway of NF-κB activation, as NF-κB inhibitors parthenolide and curcumin are known to inhibit specifically this pathway.

As has been noted earlier, it is now well documented that CS exposure may increase the incidence of diseases, like lung cancer and respiratory infections (5). Among the various immune cells, macrophages play a central role in conferring a first line defense against invading pathogens. Inflammatory mediators secreted by activated macrophages play a critical role in the protective function against exogenous stimuli. We found that differentiated monocytic cells (THP1-MΦ) secreted spontaneously high amounts of IL-8, and CS exposure did not affect it to any further extent (data not shown). Studies have shown that increased cigarette smoking intensity is associated with dose-dependent increases in BALF macrophage number and in the concentration of IL-8 in BALF (26). BALF macrophages in smokers are exposed repeatedly to CS, and this may affect IL-8 secretion.

**CS exposure affects the differentiation of monocytic cells**

CS exposure affects the differentiation of monocytic cells as indicated by the change in morphology and in CD14 expression of monocytic cells exposed to CS (Figs. 6 and 7). CD14 is involved in recognition as well as phagocytosis of whole, heat-killed bacteria. Thus, impaired CD14 expression may impair the inflammatory response of macrophages, which is crucial for keeping an infection under control. Impairment of monocytic differentiation is likely to decrease the contribution of macrophages to host defense. Deregression in differentiation may also predispose cells to malignant transformation (29). Interference with monocytic cell differentiation together with an increase in pro-inflammatory activity via NF-κB activation may play a role in the carcinogenic effects of CS. Tumor growth and metatases depend on neovascularization. Tumor neovascularization is regulated in part by monocyes and macrophages (30). Tumor fibrosis exhibit significant monocyte infiltrates, which are actively recruited to the tumor microenvironment. In tumor sites, macrophages release a number of potent proangiogenic cytokines, among them, IL-8 (31). NF-κB activation in tumor-associated leukocytes,
especially macrophages, may contribute toward tumorigenesis by upregulating tumor-promoting proinflammatory proteins (29). NF-kB activation in pre-malignant cells contributes to cell survival and metastatic potential. We believe the results of our study contribute to an understanding of the effects of human monocytic cells, exposed to CS, on inflammatory processes and tumorigenesis in bronchoalveolar airways of human lungs.

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