Nicotine Modulates the Release of Inflammatory Cytokines and Expression of TLR2, TLR4 of Cord Blood Mononuclear Cells

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ABSTRACT

The underlying mechanisms of how nicotine affects cord umbilical cells remain largely elusive. Nicotine rapidly crosses the blood-brain barrier (10 to 20 s) and binds to nicotinic acetylcholine receptors (nAChRs). Nicotine considered as a major compound found in cigarette smoke and the mechanism of nicotine action in immune response is not well understood. Cigarette smoke well known by activation of toll like receptors (TLRs) especially TLR4 and 9 which stimulates the immune response by induction of releases of cytokines mainly CXCL-8 which in turn triggers lungs reactions specially induction of neutrophils recruitments.

In this study we isolated human umbilical mononuclear cells (UCBMC) from umbilical cord blood and exposed to the nicotine for detection any cytokines and TLRs modulation.

We have found that nicotine (at concentration 0.01µM) induced release of TNF-α and IL-6 but not CXCL-8 production.

Besides we have shown that nicotine did not effect on TLR4 surface expression however up-regulated the TLR2 surface expression. Moreover expression of CD11a and CXCR4 after nicotine incubation was upregulated as demonstrated by flow cytometry analysis, These data indicated that nicotine by stimulation of inflammatory cytokines induces immune response. The present study provides evidence that nicotine selectively regulates the release of cytokines and expression of TLRs. Further studies are needed to exploring details of its effects and signaling.

Keywords: Cytokines; Nicotine; TLRs; Umbilical mononuclear cells

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INTRODUCTION

Cigarette smoking has been implicated in numerous diseases, including coronary artery disease. Cigarette smoke playing important role in the pathogenesis of diseases such as chronic obstructive pulmonary diseases and cancers. It has been shown that cigarette smoke by activation of Toll like receptors such as TLR4, TLR9 playing important role increasing cytokines such as CXCL-8 from inflammatory cells such as epithelial cells and macrophages. Nicotine is a predominant chemical among >100 constituents in cigarette smoke and has been suspected to be a causative agent for atherosclerosis for decades. The mechanism of the proatherogenic action of nicotine is still largely speculative. Moreover, nicotine augments the synthesis and secretion of proinflammatory cytokines and iNOS and the generated oxidative stress by monocytes and macrophages. Nicotine, a major constituent of cigarette smoke and also a selective cholinergic agonist, has been demonstrated to inhibit systemic inflammation. Nicotine blocks endothelial cell activation and leucocyte recruitment, suppresses dendritic cell function leading to Th1 priming and inhibits LPS induced cardiac apoptosis. Much research has been accumulated to show that nicotine alters gene expression in endothelial cells and one of the most important molecules which down-regulated by nicotine is NF-κB. However, the regulatory pathways involved in human macrophages/monocytes remain to be defined. A growing body of evidence indicates that nicotine has potent immunomodulatory actions, including suppression of Th1-type immune responses, through its interaction with the nicotinic cholinergic receptor a 7 subunit (a 7 nAChR). Consequently, nicotine exposure affects numerous systems, including neurological, neuromuscular, cardiovascular, respiratory, immunological and gastrointestinal.

Umbilical cord blood (UCB) cells are the most prevalent stem cell source available, yet have not been fully tested reactivity with nicotine. Thus in this study the objective was to determine the effectiveness of nicotine on the release of inflammatory cytokines and TLRs expression.

Herein, studies were performed to evaluate the responses and beneficial effect of mononuclear cells (MNCs) isolated from human umbilical cord blood upon nicotine incubation. Nicotine (at concentration 0.01 µM) induces release of TNF-α and IL-6 but not CXCL-8 production.

Besides the current data indicate that nicotine does not effect on TLR4 surface expression however up-regulate the TLR2 surface expression. Moreover, we show that expression of CD11a and CXCR4 after nicotine incubation were upregulated as demonstrated by flow cytometry analysis.

MATERIALS AND METHODS

Reagents
Nicotine was purchased from Sigma (St Louis, MO, USA) and 10^{-3} M solution was prepared in phosphate-buffer saline (PBS), neutralized to pH 7.2 with HCl, which was prepared freshly prior to each experiment. LPS was also purchased from Sigma and 1 mg/mL stock solution was prepared in PBS. This study was approved by an institutional review board (IRB) of Dr. Masih Daneshvari Hospital, Tehran, Iran.

The Cell Activation
Umbilical cords of full-term newborn were cleaned of maternal blood, and cord blood samples were collected under sterile conditions by puncture of the umbilical vein. UCBMC were separated from heparinized whole-blood samples by density centrifugation on Ficoll-Hypaque (Sigma Chemical Co., St. Louis, Mo., USA). The isolated mononuclear cells were washed twice in sterile phosphate buffered saline (PBS) and once in RPMI 1640 (JRH Biosciences, Lenexa, Kans., UK) and maintained in RPMI 1640 supplemented with 20% heat-inactivated, low-endotoxin fetal bovine serum (JRH Biosciences), 1 mM L-glutamine, 100 U of penicillin per mL, and 100 µg/mL streptomycin (Life Technology, Breda, The Netherlands), (basic medium).

Then cells were activated with different concentration of nicotine (Sigma-Aldrich, USA) for 24 hours. Cell viability was determined by trypan blue dye exclusion. Wright/Giemsa differential blood stains were performed on UCBMC samples.

Flow Cytometric Analysis for TLR2, TLR4, CD49d, CD11a, CD62L, CD18 and CD184 (CXCR4) Molecules Expression
Changes in the expression of human leukocyte antigens, TLR2 and TLR4, on monocytes were examined by multicolor flow cytometry using a...
combination of anti human TLR2 and anti human-TLR4 Abs. UCBMC at 1x10^6 cells/mL were incubated with nicotine at various concentrations for 24 h. Cultured cells at 5x 10^5 cells/mL were prepared for flow cytometric analysis, as described previously.19

Briefly, UCBMC cells were treated with nicotine (0.01, 1 and 100 µM) for 24 h and then washed and incubated on ice for 30 min with a PE-conjugated anti-human TLR4 (clone HTA125) or mouse IgG2a as control isotype (eBioscience, USA). TLR4 expression was assessed on a FACSscan flow cytometer (BD Biosciences). The relative TLR4 levels were quantified by the mean fluorescent intensity (MFI) from the MFI values of isotype matched control for each sample and analyzed with a FACSCalibur (BD Biosciences, San Jose, CA, USA).

For staining the adhesion molecules, cells were strained with PE-anti human CD49d, FITC-anti human CD18, FITC- anti human CD11a, PE-anti humanCD62L and PE CY7 anti-human CXCR4 for 30 min and then 10,000 cells were analyzed by FACSscan (Becton Dickinson, USA) flow cytometer. The data were processed using the CELL QUEST program.

**ELISA Assays**

UCBMC at 1x10^6 cells/mL were used for analyzing TNF-α, IL-6 and CXCL-8 production. After culturing for 24 h at 37°C in a 5% CO2/air mixture, cell-free supernatant was assayed for TNF-α, IL-6 and CXCL-8 protein by ELISA from R&D Systems, Minneapolis, MN, USA). The detection limits of the ELISA for TNF-α, IL-6 and CXCL-8 were 10 pg/mL.

**Statistical Analysis**

All data were tested for normality by IBM SPSS Statistics version 22 (IBM Corp., Armonk, N.Y., USA) and analyzed for significance using analysis ANOVA followed by Duncan test. A probability value of less than 0.05 was considered to indicate statistical significance. The results were expressed as means ±SEM of triplicate findings from five donors. The graphs were plotted using GraphPad Prism 6 (GraphPad Software, Inc., San Diego, CA).

**RESULTS**

**Effects of Nicotine on the Production of TNF-α, IL-6 and CXCL-8 by UCBMC**

As shown in Figure 1, we showed the effect of nicotine on the release of TNF-α, IL-6 and CXCL-8 protein on UCBMC. Nicotine at concentration of 0.01 µM induces release of IL-6 and TNF-α (Figure 1A and B) but no effects were seen on CXCL-8 (Figure 1C). Interestingly, no significant increase in release of those cytokines was observed by high doses (Figure 1).

Moreover, no effects on CXCL-8 were detected at various concentrations (Figure 1C).

**Effects of Nicotine on Expression of TLR2 and TLR4 by UCBMC**

In UCBMC cells, nicotine did not effect on surface expression of TLR4 (Figure 2A). LPS as a positive control down regulates TLR4 surface expressions Nicotine at concentration 0.01 µM up-regulates the surface expression of TLR2 but not at higher concentration (Figure 2B).

**Effects of Nicotine on Expression of Adhesion Molecules by UCBMC**

As shown in table.1 nicotine at concentration of 0.01, 1 and 100 µM significantly upregulates surface expression of CD11a (Table.1, p<0.05).

Nicotine at concentration of 1 and 100 µM upregulated the surface expression of CXCR4 but not at lower concentration (Table 1).

**DISCUSSION**

Toll like receptors (TLRs) are found on the cell surface and in endosomes of many different cell types. To date, 13 TLRs have been identified in mice and humans with corresponding synthetic or naturally occurring ligands. TLR4 which recognizes lipopolysaccharides (LPS) from gram negative bacteria and TLR2 which recognizes the peptidoglycan of gram-positive bacteria.20,22 We have demonstrated earlier that cigarette smoke extract induces CXCL-8 production via TLR4 in macrophage derived monocytes.6,19,22 Interestingly; this effect was not due to contamination of LPS.3,4

It has been demonstrated that maternal smoking and the levels of nicotine in blood have many side effects on the growth and neurological development.23-27 On the other hand nicotine considered as an important composition of cigarette smoke, thus in the current study we aimed to investigate the effects of nicotine on the UCBMC as a cells which directly affected by maternal smoke status.

We have found that nicotine at concentration of
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Figure 1. The effect of nicotine on the production of IL-6, chemokine (C-X-C motif) ligand (CXCL)-8 and TNF-α by UCBMC cells.

Umbilical cord blood mononuclear cells (UCBMC) cells were activated with various concentrations of nicotine for overnight, after that the cells were centrifuged and supernatants were used for ELISA analysis for release of cytokines. A) Nicotine at concentrations of 0.01 and 0.1 µM increases the release of TNF-α (p≤0.01), B) shows release of IL-6 and as indicated only at 0.01 µM increases of release of IL-6 (p≤0.01). C) Shows lack of release of CXCL-8. None of the concentrations of nicotine induced release of CXCL-8.

LPS served as a positive control for releases of cytokines.

Figure 2. Modulation of TLR4 expression by nicotine

Umbilical cord blood mononuclear cells (UCBMC) cells were treated with nicotine (0.01, 0.1 and 1 µM) for 24h and then incubated with phycoerythrin (PE) conjugated anti-toll like receptor (TLR) 4, fluorescein isothiocyanate (FITC) conjugated anti-TLR2 or isotype controls antibody as described in materials and methods section. Fluorescence-activated cell sorting (FACS) analysis of a representative of at least 3 experiments showing the mean fluorescence intensity (MFI) difference of each group. Values are expressed as mean+/−S.E.M (n= 3). *p= 0.05, ***p= 0.001 significantly different compared to control.
Table 1. Effects of Nicotine on expression of adhesion molecules on UCBMC cells

<table>
<thead>
<tr>
<th>Markers</th>
<th>Control</th>
<th>LPS (1 µg/mL)</th>
<th>Nico (0.01 µM)</th>
<th>Nico (1 µM)</th>
<th>Nico (100 µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CD49d</td>
<td>39.6±1.5</td>
<td>47±7.5</td>
<td>46±6</td>
<td>46±5.5</td>
<td>48.3±4</td>
</tr>
<tr>
<td>CD18</td>
<td>41.3±2.02</td>
<td>50±2.88*</td>
<td>1.73±40</td>
<td>1.2±36.6</td>
<td>35.6±1.45</td>
</tr>
<tr>
<td>CD11a</td>
<td>48±2.08</td>
<td>58.6±4.25</td>
<td>65.6±4.4*</td>
<td>60.6±6.35*</td>
<td>67.3±7.8*</td>
</tr>
<tr>
<td>CD62L</td>
<td>56±3.51</td>
<td>50.6±3.21</td>
<td>56.3±2.3</td>
<td>57±2.3</td>
<td>58.6±2.6</td>
</tr>
<tr>
<td>CD184(CXCR4)</td>
<td>26±1.52</td>
<td>35.3±3.38</td>
<td>34.3±5.36</td>
<td>39±3.21*</td>
<td>40±2.08*</td>
</tr>
</tbody>
</table>

Umbilical cord blood mononuclear cells (UCBMC) were treated with nicotine (0.01, 0.1 and 1µM) for 24h and then incubated with PE conjugated anti-CD49d, FITC conjugated anti-CD18, Fluorescein isothiocyanate (FITS) conjugated anti-CD11a, PE conjugated anti-CD62L and PE.CY7 conjugated anti-CXCR4 for 30 min. After washing 10000 cells were analysed by FACS Calibour. The percent of expression of above markers were plotted in table.

0.01 µM increased the release of TNF-α and IL-6 such as LPS, however we did not find any regulation of CXCL-8 by nicotine in UCBMC.

At lower concentration of nicotine less than 0.01 µM, no effects of nicotine was either found on cytokine releases and TLRs regulation (data not shown).

Moreover, we have found that nicotine regulates surface expression of TLR2 but not TLR4 in UCBMC. Interestingly, we have found that nicotine did not affect surface expression of TLR4 however LPS down regulate this expression. Not much data available about the effectiveness of nicotine in regulation of TLRs expression and function. To our knowledge this is the first report of effects of nicotine on expression of TLRs. Further studies need to show in details of role of TLRs with nicotine and function.

The surface expression of TLR2 by nicotine at low concentration (0.01 µM) was up-regulated however at 1 and 100 µM did not affect. The effectiveness of nicotine for regulation of TLRs is new finding and needs to be elicited.

Interestingly, nicotine upregulates the surface expression of CD11a and CXCR4 in UCBMC cells. In correspondence, it has been reported that nicotine induces mononuclear leukocyte adhesion and expression of adhesion molecules such as VCAM and ICAM, in endothelial cells in vitro. Moreover, nicotine exerts inhibitory effects on both endothelial cell surface intercellular adhesion molecule expression and neutrophil integrin expressions of CD62L, CD11a, and CD11b in vitro.26 It has suggested that these in vitro effects of nicotine may relate to the clinical observation of reduced incidence of preeclampsia in women that smoke.

The novel finding that nicotine augments proinflammatory cytokine synthesis in UCBMC is the key entry point for understanding the molecular mechanism of nicotine-augmented inflammatory response in the fetus. Research in the relationship between tobacco use and adhesion molecule networks is at an early stage. Adhesion molecules are, central and critical components of the immune and inflammatory system.

CXCR4, also known as “fusin,” is one of the most well-studied chemokine receptors due to its earlier finding role as a coreceptor for HIV entry.

(28). The chemokine stromal cell-derived factor-1, now renamed as CXCL12, was established as the specific ligand for CXCR4.29,30

CXCR4 is commonly expressed on most hematopoietic cell types including macrophages, monocytes, T and B lymphocytes, neutrophils, hematopoietic, endothelial progenitor, and stem cells in the blood or bone marrow, dendritic cells and Langerhans cells.31-33 The CXCR4/CXCL12 axis is indispensable for cell migration during embryonic
hematopoiesis, organogenesis, vascularization, and organ homeostasis.\textsuperscript{34}

The CXCR4/CXCL12 axis also plays an important role in inflammation and immune surveillance of tissues.\textsuperscript{35}

Role of CXCR4 in autoimmune and inflammatory diseases can be found elsewhere.\textsuperscript{36} Thus, in the current study upregulation of CXCR4 receptor by nicotine may have impact on the immunological function of UCBMC. The current study had few limitation including finding smokers and nonsmokers of UCBMC and determination blood levels of nicotine in blood of smokers.

In conclusion, our study has enhanced understanding of the molecular steps leading to nicotine augmentation of inflammatory cytokines by UCBMC and provides additional rationale for application of anti-inflammatory therapeutic approaches for smokers for prevention and treatment of smokers and individuals with long-term nicotine usage.

REFERENCES