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Cigarette smoke condensate affects monocyte interaction with endothelium

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ABSTRACT

Objective: Circulating monocytes adhere to the endothelium and migrate into the intima contributing to atherosclerotic plaque growth. Cigarette smoke is a risk factor for atherosclerosis, but it is not completely known how it affects monocyte behavior in atherogenesis.
Methods: We studied the effects of cigarette smoke condensate (CSC) on human monocytes (HM) chemotaxis and transmigration through an endothelial cell (EC) monolayer.
Results: Pre-treatment with CSC caused a decrease in HM chemotaxis and transmigration (−55% and −18% vs control, $p < 0.05$, respectively), paralleled by a reduced expression of Rac 1 GTPase. On the contrary, direct exposure of both HM and EC to CSC increased (+23% vs control, $p < 0.05$) HM transmigration, paralleled by a strong stimulation of VCAM1 and ICAM1 expression by ECs, and by a slight increase in monocyte integrin expression. An enhancement of monocyte transmigration was obtained after the exposure of both HM and EC to medium conditioned by HM previously incubated with CSC (+265% vs control, $p < 0.001$). CSC showed a stimulatory effect on the expression by HM of TLR4, MCP1, IL8, IL1beta, and TNFalfa, which was ablated by pre treatment with PDTC. Incubation with neutralizing antibodies against both MCP1 or IL8 completely abolished the CSC-conditioned medium induced HM transmigration.
Conclusions: CSC induces HM to release chemotactic factor(s), which amplify the recruitment and transmigration of inflammatory cells through EC, but CSC may also reduce HM migratory capacity. Therefore, exposure to CSC affects monocyte behavior and interaction with the endothelium, thus potentially facilitating and/or further aggravating the atherogenic process.
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1. Introduction

Cardiovascular disease (CVD) is the leading cause of mortality in developed countries and is likely to attain this status worldwide, accounting for 17.3 million deaths each year [1]. Environmental factors may contribute to CVD risk, incidence, and severity [2]. Among these, the incidence of cigarette smoking is now increasing rapidly throughout the developing world and is one of the biggest threats to current and future world health [3]. While the adverse effects of cigarette smoke on lung health are well established, it is becoming evident that smoke has an important extra-pulmonary toxicity [4]. Epidemiological studies have shown that cigarette smoking is a major risk factor for atherosclerosis and related diseases such as CVD [5] and tobacco use is one of the major avoidable causes of CVD [6]. In animal models of exposure, tobacco smoke induces pro-thrombotic responses and exacerbates atherogenesis and myocardial ischemic injury [2]. Tobacco smoke interacts with inflammatory cytokines to produce endothelial dysfunction [7] and induces pro-inflammatory and pro-atherosclerotic effects in vascular tissue [8]. Moreover, recent studies have demonstrated a statistically significant reduction in acute coronary events in the adult population after the introduction of a smoking ban throughout Italy [9], an effect that was consistent with the pollution reduction observed in indoor public places and with the known deleterious effects of passive smoking on health.

Whole cigarette smoke is a complex aerosol mixture made up of over 6000 chemical components distributed between the particulate and gas phases, including nicotine, aromatic hydrocarbons, sterols and oxygenated isoprenoid compounds, aldehydes, nitriles, cyclic ethers, and sulfur compounds [10,11]. Although cigarette smoke is widely accepted as an environmental factor that aggravates atherosclerosis, less well known are the mechanism(s) at the

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basis of its deleterious effects and which smoke component(s) are involved in the major pathophysiological effects.

Inflammation characterizes atherogenesis and links many traditional risk factors to altered function of arteries [12]. Monocytes/macrophages have a key role in the initiation and progression of atherosclerotic plaques and the accumulation of lipid-laden macrophages characterizes the plaque. In the initial phase of plaque development, a pro-inflammatory milieu stimulate vascular endothelial cells (EC) to express adhesion molecules and chemoattractants that recruit inflammatory monocytes into the vascular wall. Blood monocytes express integrins that mediate monocyte adhesion to the dysfunctional endothelial surface by binding to adhesion molecules. Pro-inflammatory proteins known as chemokines provide a chemotactic stimulus that induce monocytes to enter the intima [12]. Toll-like receptors (TLRs) can directly affect atheroma formation as stimulation of macrophages with TLR2, TLR4 and TLR9 ligands promote lipid uptake [13]. Moreover, activation of TLRs triggers a signaling cascade via MyD88 and NF-kB activation which increases the transcription of pro-inflammatory cytokines that contribute to local inflammation and growth of the plaque [13]. Several chemokines may recruit leukocytes into the arterial intima [14], and interleukin 8 (IL8) and monocyte chemoattractant protein 1 (MCP1) appear to play an early and important role in the recruitment of monocytes to atherosclerotic lesions and in the formation of intimal hyperplasia after vessel wall injury [15,16]. Once resident in the arterial intima, monocytes differentiate to macrophages that can further exacerbate the local inflammation through the production of MCP1 or IL8, and TNFα or IL1β, that promote recruitment and activation of additional immune cells [17]. In addition, many monocyte-derived cells may migrate out from atherosclerotic lesions and this step characterizes regressive, but not progressive, atherosclerotic plaques [18]. Thus, progression of atherosclerotic plaques may result not only from robust monocyte recruitment into arterial walls but also from reduced emigration of these cells from lesions [18].

Based on the above premises, in order to gain insights into the effect(s) of cigarette smoke on monocytes/macrophages and endothelial cells functions, we studied and examined the effects of CSC on human monocytes (HM) *in vitro*. In particular, we analyzed HM chemotaxis induced by different chemotactic factors and HM interaction with and transmigration through an EC monolayer. Moreover, we evaluated the effect(s) of CSC on the expression of chemotactic inflammatory cytokines by HM.

2. Methods (for more details, please see the Supplementary materials)

2.1. Cigarette smoke condensate

Cigarette smoke condensate (CSC) was provided by British American Tobacco (Southampton, UK). CSC was dissolved in dimethylsulphoxide (DMSO) to yield a final concentration of 24 mg/mL. Final maximal DMSO concentration in all samples was adjusted to 0.1%.

2.2. Cell culture

The study was performed conform to the declaration of Helsinki. Approval for this study was granted by the University of Milan ethics review board.

Human monocytes (HM) were isolated from the blood of healthy, non-smokers volunteers as described [19], using a Ficoll–Paque gradient (GE Healthcare). The final pellet was suspended in serum-free Dulbecco's modified Eagle's Medium (DMEM) (Euroclone), and cells were plated at a density of 3×10^6 cells/well in 6 well plates. HM were isolated by cell adhesion to culture dishes for 1 h.

The human endothelial cell line EAhy.926 [20,21] was cultured in DMEM supplemented with 10% FCS, 1% HAT (hypoxanthine-aminopterin-thymidine medium), streptomycin and penicillin. (For more details on this cell line, please see Supplementary materials).

For the experiments, HM were incubated for 24 h at 37 °C with DMEM supplemented with 0.2% essential fatty acid free albumin (EFAF) and increasing concentrations of CSC. To investigate the signal transduction pathway involved in CSC induced effects, cells were treated with an inhibitor of NF-kB, pyrrolidine dithiocarbamate (PDTC 10 μM; Sigma), for 1 h, and then CSC (30 μg/ml) or DMSO were added for 24 h before RNA isolation.

Conditioned medium was prepared by incubating HM for 24 h at 37 °C with DMEM containing CSC 30 μg/ml. Then cells were spun down and medium kept at −80 °C until use.

For the experiments with neutralizing antibodies, conditioned medium was incubated for 1 h at 37 °C with neutralizing antibodies against IL8 (2.5 μg/ml) or MCP1 (0.1 μg/ml), or irrelevant IgG1 as control. Then, these media were added to the lower part of the Boyden chamber and HM migration was assessed as described in supplementary materials section.

2.3. Monocyte adhesion assay

Adhesion assay was performed using the fluorescent dye 2′,7′-Bis(2-carboxyethyl)-5(6)-carboxyfluorescein tetrakis(acetoxymethyl) (BCECF-AM). Endothelial cells were seeded in 6-well plates. After reaching confluence, EC were activated with TNFα (10 ng/ml) for 48 h. Monocytes were fluorescently labeled with BCECF-AM, added to the EC monolayer (2×10^6 cells/ml) and incubated for 1 h at 37 °C. Media were carefully removed and cells were gently washed twice with PBS and lysed with 4% solution of Triton X-100. Fluorescence was quantified using a Fluoroskan with BCECF's excitation maximum of 485 nm and emission maximum of 535 nm.

2.4. Monocyte transmigration assay

Monocyte transmigration was quantified using the CytoSelect Leukocyte Transmigration Assay (Cell Biolabs, Inc. San Diego, CA. USA) according to manufacturer's instructions. EC were seeded in the inserts of 24-well transwell plates. After 72 h, cells were activated with TNFα (10 ng/ml) for 48 h and fluorescence-labeled monocytes were added on top of EC and incubated for 6 h. Media were carefully removed from the transwells and non-migrated cells were gently removed using cotton swabs. The swabbed transwells and the media from the underside of the transwell inserts were transferred to clean wells containing lysis buffer and incubated for 5 min at room temperature. Lysates were then transferred to a 96-well plate for fluorescence measurement.

2.5. Quantitative Real-Time PCR (QRT-PCR)

Total RNA was extracted with EuroGOLD Trifast™ solution (Euroclone) and processed for reverse transcription (BIORAD). The mRNA levels for specific genes were determined by QRT-PCR, using 20 ng of total cDNA amplified with 1× SYBR Green mastermix (BIORAD). Each sample was analyzed in triplicate using the ABI PRISM 7000 Sequence detection system. The primers for IL8, IL1β, TNFα, MCP1, VCAM1, ICAM1, LFA-1, VLA-4, and TLR4 were purchased from PRIMM srl (Milan, Italy). All data were normalized to β-actin content and expressed as fold changes over control.

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