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Mechanism of an indirect effect of aqueous cigarette smoke extract on the adhesion of monocytic cells to endothelial cells in an *in vitro* assay revealed by transcriptomics analysis

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ABSTRACT

The adhesion of monocytic cells to the "dysfunctional" endothelium constitutes a critical step in the initiation of atherosclerosis. Cigarette smoke (CS) has been shown to contribute to this process, the complex mechanism of which still needs to be unraveled. We developed an *in vitro* adhesion assay to investigate the CS-induced adhesion of monocytic MM6 cells to human umbilical vein endothelial cells (HUVECs) following exposure to an aqueous CS extract (smoke-bubbled phosphate buffered saline: sbPBS), reasoning that *in vivo* monocytes and endothelial cells are exposed primarily to soluble constituents from inhaled CS absorbed through the lung alveolar wall. MM6 cell adhesion was increased exclusively by the conditioned medium from sbPBS-exposed MM6 cells, not by direct sbPBS exposure of the HUVECs within a range of sbPBS doses. Using a transcriptomics approach followed by confirmation experiments, we identified different exposure effects on both cell types and a key mechanism by which sbPBS promoted the adhesion of MM6 cells to HUVECs. While sbPBS provoked a strong oxidative stress response in both cell types, the expression of E-selectin, VCAM-1 and ICAM-1, responsible for the adhesion of MM6 cells to HUVECs, was induced in the latter through a proinflammatory paracrine effect. We confirmed that this effect was driven mainly by TNFα produced by MM6 cells exposed to sbPBS. In conclusion, we have elucidated an indirect mechanism by which sbPBS increases the adhesion of monocytic cells to endothelial cells in this *in vitro* assay that was designed for tobacco product risk assessment while mimicking the *in vivo* exposure conditions as closely as possible.

1. Introduction

Atherosclerosis is the main underlying risk factor for cardiovascular disease, a leading cause of deaths worldwide (Hansson, 2005; Services, 2010). Although the complex mechanisms leading to atherosclerosis are not yet fully understood, it has been recognized that the increased adhesion of monocytic cells to the "dysfunctional" or "activated" vascular endothelium is an early, pro-inflammatory event during the initiation of plaque formation in the vessel wall (Huang et al., 2011; Kawakami et al., 2006; Libby et al., 2002; Ross, 1999). The term "endothelial dysfunction" generally implies a state characterized by diminished production or availability of nitric oxide leading to impaired vasodilation, and a shift towards a proinflammatory, prothrombotic, pro-oxidative, and proadhesive phenotype of the endothelium, while the term "endothelial activation" often refers more specifically to the proinflammatory and proadhesive changes occurring in endothelial cells (Alom-Ruiz et al., 2008; Flammer et al., 2012; Szmitko et al., 2003; Winkelmann et al., 2009). The active recruitment of monocytes from the bloodstream by cell–cell adhesion to the luminal surface of "activated" endothelial cells at sites known to be prone to the

Abbreviations: Log_2-FC, Log_2 fold change; FDR, false discovery rate; CS, cigarette smoke; sbPBS, smoke-bubbled phosphate buffered saline; GSEA, gene set enrichment analysis; MM6, Mono Mac 6 cell line; HUVECs, human umbilical vein endothelial cells; HCAEC, human coronary arterial endothelial cells; VCAM-1, vascular cell adhesion protein 1; ICAM-1, intercellular adhesion molecule 1; TNFα, tumor necrosis factor-alpha; NRF2, Nuclear factor, erythroid derived 2, like 2; NFκB, nuclear factor-kappa B; IL1B, interleukin 1 beta; oxLDL, oxidized low-density lipoprotein; RT-qPCR, reverse transcriptase-quantitative polymerase chain reaction; NES, normalized enrichment score.

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development of atherosclerotic plaques (e.g., in the coronary and carotid arteries) is a prerequisite for the subsequent transmigration of bound monocytes across the endothelium into the intima region of the vessel wall, where the infiltrated monocytes differentiate into foam cells and give rise to fatty streaks and eventually atherosclerotic plaques (Libby, 2002).

Cigarette smoking is a well-known risk factor for the development and progression of atherosclerosis (Burns, 2003; Howard et al., 1998; Lakier, 1992; Pipe et al., 2010; Tsiara et al., 2003; Winkelmann et al., 2009). It has been repeatedly demonstrated that cigarette smoke (CS) influences the monocyte-endothelial adhesion process. The adhesion molecules vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1) and E-selectin, which play major roles in the leukocyte adhesion process and whose expression on the surface of endothelial cells can be induced by oxLDL, the acute-phase protein high sensitivity C-reactive protein, or by cytokines such as IL-1beta or TNF alpha, have been shown to be up-regulated by CS (Szmitko et al., 2003). Indeed, various *in vitro* assays have demonstrated the influence of CS or its constituents on the regulation of these adhesion molecules, sometimes with contradictory results that may in part depend on differences in cell type, CS fraction (e.g., CS condensate or aqueous extract), and/or exposure conditions (flow or steady state) (Chen et al., 2004, 2009; Kalra et al., 1994; Lehr, 1993; Scott and Palmer, 2002; Shen et al., 1996). *In vivo*, CS has been shown to induce leukocyte aggregation and adhesion to endothelium prevented by vitamin C (Lehr et al., 1994). A significant increase in ICAM-1 mRNA was observed in the coronary arteries of rats exposed to CS daily for 1 week (Csiszar et al., 2008). Interestingly, sera from smokers in which increased monocyte-endothelium adhesion was confirmed induced the up-regulation of ICAM-1 in HUVECs (Adams et al., 1997). This result suggests that the serum of smokers exhibits properties that favor the process of adhesion. Numerous population-based studies have shown that the systemic compartment of smokers, compared with that of never-smokers or ex-smokers, displays a low-grade inflammatory response with increased levels of circulating proinflammatory cytokines such as IL-6 (Bermudez et al., 2002a,b; Woodward et al., 1999). These cytokines, the systemically distributed soluble constituents and their metabolites derived from the inhaled CS (absorbed through the lung alveolar epithelium and the adjacent microvascular endothelium), or a combination of the two, may have an effect on the regulation of adhesion molecules in endothelial cells observed *in vivo*. This raises important questions regarding whether soluble CS constituents have a direct and/or indirect effect on the regulation of VCAM-1, ICAM-1 and E-selectin in endothelial cells, and the extent to which these molecules are involved in the process of monocytic cell adhesion to the endothelium. To investigate these questions, we tested the effect of medium conditioned with an aqueous CS extract in the form of smoke-bubbled phosphate buffered saline (sbPBS) in an *in vitro* adhesion assay. For this purpose, the human monocytic cell line, Mono Mac 6 (MM6), and human umbilical vein endothelial cells (HUVECs) were used as relevant cellular models to study the process of adhesion. The adhesion of MM6 cells to HUVECs using TNFα as an inducer of adhesion has previously been described by Erl et al. (Erl et al., 1995). MM6 adhesion was shown to reflect mature human monocytes because similar adhesion parameters were measured, in parallel, in isolated blood monocytes (Couffinhal et al., 1993; Erl et al., 1995). In the present study, the *in vitro* conditioned-medium and adhesion experiments combined with transcriptomics gene profiling and confirmation experiments enabled to show that sbPBS promotes the adhesion of monocytic cells to endothelial cells through an indirect effect, in which TNFα plays a prominent role.

2. Material and methods

2.1. Materials

Recombinant TNFα was purchased from R&D Systems (Minneapolis, MN, USA). The rat anti-human blocking antibody against TNFα was purchased from AbD Serotec (cat. No. MCA1560, Kidlington, UK). Murine mAbs were used to block the adhesion molecules, E-Selectin (R&D cat. No. BBA16), ICAM-1 (Abd Serotec, cat. No. MCA1615EL), and VCAM-1 (R&D cat. No. BBA5). All blocking antibodies were IgG1 isotype. The IgG1 isotype controls were purchased from AbD Serotec and R&D Systems (cat No. 5172–6004 and cat. No. MAB002). Collagen A was purchased from Biochrom (Berlin, Germany).

2.2. Cell culture

Human umbilical vein endothelial cells (HUVECs) were purchased from PromoCell (Heidelberg, Germany) and cultured in endothelial growth medium containing supplement mix on collagen A-coated cell culture dishes. Experiments were performed at subculture 2–8 using 1-day post-confluent cells. The human monocytic Mono Mac 6 (MM6) cell line was obtained from Prof. Ziegler-Heitbrock (University of Leicester, UK). The cells were maintained in RPMI 1640 containing 10% (vol/vol) fetal calf serum (FCS), 2 mM L-glutamine, non-essential amino acids (PAA Laboratories, Pasching, Austria), 1 mM sodium pyruvic acid, and 9 mg of bovine insulin (Sigma–Aldrich, Deisenhofen, Germany).

2.3. Preparation of aqueous CS extract, i.e., smoke-bubbled phosphate buffered saline (sbPBS)

Mainstream smoke from the Reference Cigarette 3R4F (University of Kentucky) was generated on a 20-port Borgwaldt smoking machine according to ISO standard 3308 and bubbled through PBS (10 cigarettes/32 mL PBS, ~2.5 puffs/mL) at room temperature as previously described (Muller, 1995; Muller and Gebel, 1994). This stock solution was diluted in cell culture medium (0.5% FCS) to obtain the final sbPBS concentrations varying from 0.015 to 0.18 puffs/mL. Only freshly prepared sbPBS was used in the experiments.

2.4. Preparation of conditioned-media

MM6 cells (2×10^6) were cultured in 6-well culture plates in standard medium (RPMI 1640 supplemented with 0.5% with FCS 2 h prior treatment with sbPBS). Cells were exposed to several doses of sbPBS or PBS (solvent control) diluted in starved standard medium (RPMI1640 with 0.5% FCS) at 37 °C for 2 h. Subsequently, the supernatants and lysates of MM6 cells were collected and stored at −80 °C for the adhesion assay and RNA extraction (Fig. S1). In parallel, PBS (solvent control) and sbPBS solutions were diluted in starved standard medium (RPMI1640 with 0.5% FCS), incubated without any MM6 cell at 37 °C for 2 h, and then stored at −80 °C for the adhesion assay.

2.5. Treatment of HUVECs or HCAECs with conditioned-medium

HUVECs or HCAECs were grown as monolayers in collagen-coated 48-well culture plates until confluent in endothelial growth culture medium from PromoCell (Heidelberg, Germany). Cells were then starved overnight in the same medium containing 0.5% FCS and incubated for 4 h with the supernatant from MM6 cells exposed to sbPBS or PBS (indirect treatment). In parallel, HUVECs and HCAECs were exposed directly to PBS or sbPBS diluted in