

Does Exposure to Cigarette Smoke Compromise Mast Cell Function?: Implications for Chronic Lung Inflammation and Host Defense Against Pathogens

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Citation

I Orouk, L Bellehsen, F Levi-Shaffer. *Does Exposure to Cigarette Smoke Compromise Mast Cell Function?: Implications for Chronic Lung Inflammation and Host Defense Against Pathogens*. The Internet Journal of Asthma, Allergy and Immunology. 2006 Volume 6 Number 1.

Abstract

Cigarette smoke (CS) is linked to increased frequency of pulmonary infections, airway hyper-reactivity & chronic inflammation. We investigated effects of soluble CS components (CSE) on mast cells (MC), which are principal effectors in these diseases. Cord blood MC (CBMC) were isolated and cultured with stem cell factor (SCF), prostaglandin (PG) E₂ and interleukin (IL)-6 to derive primary lines and then exposed to varying concentrations (0-10%) of CSE for intervals of 12-48h. Viability and MC status before and after IgE-dependent- and independent (compound 48/80) - activation were determined by Trypan Blue exclusion, by β -hexosaminidase (β -hex) release and mitogen-activated protein kinase (MAPK) phosphorylation. CSE at 2.5% was non-toxic and significantly increased MC degranulation following activation. To evaluate indirect effects of CSE, CBMCs were co-cultured with 3T3 fibroblasts and β -hex release and expression of Gi3 (which is associated with granule exocytosis and eicosanoid generation) was measured in MC. CSE inhibited degranulation and Gi3 expression in a contact-dependent fashion. CSE also altered phosphorylation of ERK1/2, p38, and JNK after activation. Thus, CS may modulate MC function in airway inflammation. representative

This study was supported by funds from the Aimwell Charitable Trust (UK).

INTRODUCTION

Mast cells (MC) originate from pluripotent haematopoietic bone marrow stem cells that give rise to all leukocytes in the body [1]. Unlike other haematopoietic cells that leave the bone marrow as fully mature cells, MCs leave the bone marrow as immature committed progenitors and undergo their final differentiation in connective tissues such as the skin, and in the mucosae of the respiratory tract and gut under the influence of stem cell factor (SCF) and other locally produced cytokines. The interaction between SCF and its receptor, c-Kit provides the most important viability and differentiation signal for MC [1, 2]. Other mediators such as interleukin (IL)-3, IL-4, IL-9 and IL-10, nerve growth factor (NGF), some chemokines and retinoids also regulate MC differentiation [3]. The number of MCs within connective tissue is normally constant, whereas their numbers in the respiratory and gastrointestinal tracts can vary considerably. In inflammatory conditions such as allergy, asthma, rheumatoid arthritis (RA) and inflammatory

bowel disease MC numbers may increase markedly in the affected tissue [4,5,6,7,8]. Asthmatic patients also have more circulating immature progenitors that have the potential to differentiate into mature MCs [9].

MC may be activated immunologically by cross-linking of multivalent antigen (allergen) with specific IgE antibody attached to the MC membrane via its high affinity receptor, Fc ϵ RI. Cross linking of IgE by the interaction of allergen with specific determinants on its Fab portion results in activation with subsequent release of stored mediators and de novo synthesis of others. MC can also be activated non-immunologically by polybasic molecules such as compound 48/80, substance P, and anaphylatoxins derived from split complement components (C3a, C4a, and C5a) [10]. These cause MC exocytosis by directly activating Gi3, a pertussis toxin-sensitive Gi protein that controls granule movement [11, 12]. Upon activation MC can release a wide variety of multifunctional mediators. These include: pre-formed mediators such as histamine, proteases and proteoglycans that are stored in cytoplasmic granules and are rapidly released upon activation; lipid-derived mediators such as

leukotrienes (LT) and prostaglandins (in particular, PGD₂) which are synthesized de novo via the arachidonic pathway and secreted later; and a variety of cytokines which include interleukins, tumor necrosis factor (TNF)- α ; transforming growth factor (TGF)- β and chemokines (CCL3 and CXCL8). These are not only important in orchestrating chronic inflammation but also influence the development of innate and acquired immunity. MCs are involved in host defense [13]. MCs function as antigen-presenting cells as they have the capacity to phagocytose diverse pathogens and to express MHC Class I and -II molecules as well as co-stimulatory molecules, allowing them to interact with endothelial cells, T- and B-lymphocytes [14, 15]. These interactions amplify IgE production by B-cells, T-cell proliferation and cytokine release thereby promoting allergic inflammation. Furthermore, MCs can release chemotactic factors (chemokines) that attract lymphocytes and other inflammatory cells to inflamed or injured tissues in conditions such as asthma [16].

While other inflammatory cells such as eosinophils, T-lymphocytes, IgE-producing B-cells, and neutrophils are clearly involved in the pathogenesis of asthma [17]. Due to their unique location in all vascularized tissues including the bronchi, and the mediators they release, MCs play a key role. Histamine contributes to bronchoconstriction, vasodilatation and tissue edema in asthma [18]. MC-derived LTs and PGD₂ [19] are also powerful bronchoconstrictors and vasodilators and synergize with MC tryptase, and cytokines in promoting leukocyte infiltration [4, 20]. MC components may also contribute to chronic inflammatory changes that compromise lung function in asthma and other conditions. MC hyperplasia and degranulation have been observed in fibrotic diseases, such as idiopathic lung fibrosis, chronic asthma, Crohn's disease, and scleroderma [3]. MC-derived vascular endothelial growth factor (VEGF) and fibroblast growth factor (FGF)- β are not only important in development of fibrosis but also in angiogenesis and have been associated with de novo vascularization and lung remodeling. [21,22].

Tobacco smoke is a transient respiratory irritant that has been associated with lung cancer, chronic obstructive pulmonary disease (chronic bronchitis and emphysema), increased airway reactivity [23], exacerbation of asthma [24], and increased frequency of pulmonary infections [25]. Cigarette smoke (CS) contains over 4,000 currently identified chemicals, of which many have been examined and found to be biologically active in both short- and long-

term exposures. Among the chemically defined constituents are gaseous nitrosamines, aromatic polycarbons, aldehydes and heavy metals [25]. Although it is clear that exposure to CS promotes asthma-related morbidity and increases mortality [26, 27], its role in airway inflammation and remodeling in asthma remains largely undefined. However, various clinical studies have implicated increased MC numbers and their mediators in lung pathology [28,29,30,31,32,33] and studies in rodent models have shown that CS-exposure induces chronic inflammation in the lung associated with development of emphysema, lung remodelling, and decreased local immunity [34,35,36,37,38,39,40]. As a first step to understanding the role of MC in CS-induced inflammation we performed in vitro studies to determine the impact of soluble components of CS in the form of cigarette smoke extract (CSE) on MC survival, activation, and functional properties. In these studies we employed cord blood-derived MC (CBMC), which resemble closely mucosal MC found in the lung [41]. The results of the studies described herein suggest that CS may modulate MC function, both directly and indirectly.

MATERIALS AND METHODS

PREPARATION OF CIGARETTE SMOKE EXTRACT (CSE)

Smoke from one cigarette (Marlboro Lights, Phillip Morris, estimated to contain 10 mg tar, 0.8 mg, 11 mg carbon monoxide at the smoker end [42]) was extracted under vacuum for 5 min into 10 ml of culture medium using an apparatus designed for this purpose as previously described [43]. The solution was subsequently sterilized through a 0.45 μ m disposable filter. The cigarette smoke extract (CSE) was prepared freshly for each experiment and assigned an absolute value of 100%. For experiments with MC, CSE was diluted in culture medium to concentrations ranging from 0 to 10%.

ISOLATION OF CORD BLOOD MAST CELLS (CBMC)

MC short term lines were prepared from human cord blood. All specimens were obtained in compliance with the conventions of the Helsinki Declaration. Mononuclear cells were isolated from freshly collected heparinized human umbilical cord blood using density gradient centrifugation (Histopaque™ (density=1.077, from Sigma-Aldrich Chemicals, St. Louis, MO) according to the manufacturer's directions. The mononuclear cell layer was collected and washed twice by centrifugation. Total MCs present in this

population were determined after staining with acidified Toluidine Blue (Sigma-Aldrich) and hemocytometer counting. MCs were seeded at a density of 1×10^6 cells/ml in Minimal Essential Medium (MEM- β , Biological Industries) containing 10% FCS (v/v), 100 U/ml penicillin, 100 μ g/ml streptomycin, 2 mM L-glutamine, 10 μ g/ml ribonucleases, 100 ng/ml SCF (a generous gift from Amgen, Thousand Oaks, CA), 10 ng/ml IL-6 and 3×10^{-7} M PGE₂ (Sigma-Aldrich) and incubated at 37 ° C in 5%CO₂) in upright 200-ml culture flasks (Nunc, Denmark). Half of the culture medium was replaced every week. CBMCs were harvested for the experiments after culturing for 6-9 weeks when more than 95% of the cells stained metachromatically with Toluidine Blue.

EVALUATION OF MAST CELL VIABILITY

To determine the effect of CS on MC viability and function, CBMC (1×10^6 cells/ml) were cultured in MEM- β supplemented with 100 ng/ml of SCF. Various concentrations of CSE (0-10%) were added for intervals of 12, 24 & 48h and the cells were incubated at 37°C in 5% CO₂. MC viability was determined by Trypan Blue exclusion using a Neubauer hemocytometer.

NON-IMMUNOLOGIC ACTIVATION OF MC

CBMCs (1×10^6 cells/ml) were cultured in medium containing varying concentrations of CSE (0-5%) for 48 h (37°C, 5% CO₂), cells were washed twice with Tyrode's buffer and then incubated for 30 min under the same conditions with 10 μ g/ml of compound 48/80 (Sigma-Aldrich).

IGE-DEPENDENT ACTIVATION OF MC

CBMCs were sensitized with 5 μ g/ml of human IgE (Serotec, Oxford, UK) in culture medium for 5 days incubating at 37 ° C in 5% CO₂. After this, varying concentrations of CSE (0-5%) were added to the cells and the incubation was continued for 48 h under the same conditions. The cells were washed twice with Tyrode's buffer and then incubated with 10 μ g/ml of mouse anti-IgE (Serotec) for 30 min at 37 ° C in 5% CO₂ to activate them. In both cases activation was stopped by removing the cells from the activating solutions by centrifugation at 400g for 5 min at 4 ° C. Activation was quantitatively determined by β -hex release into the culture supernatants (see below).

MC MEDIATOR RELEASE ASSAYS

The preformed mediator, β -hexosaminidase (β -hex) was

measured in the supernatant of activated MCs by a colorimetric assay employing p-nitrophenyl N-acetyl β -D-glucosaminide (Sigma-Aldrich) as a substrate: 18 μ l of supernatant from activated MC or Tyrode's buffer (negative control) were transferred to a 96-well plate containing 42 μ l of β -hex substrate solution. The plate was incubated for 2 h at 37°C in 5% CO₂. The reaction was stopped by adding 120 μ l of 0.2M glycine (pH 10.7) to each well. Absorbance at 410 nm (OD₄₁₀) was determined spectrophotometrically using a microplate reader.

MAST CELL-FIBROBLAST CO-CULTURES

In vivo MCs interact with other cells in their microenvironment, in particular, fibroblasts. To evaluate the effects of CS on these cellular interactions, CBMCs were added to confluent monolayers of Swiss albino embryonic mouse 3T3 fibroblasts (American Type Culture Collection, Rockville, MD), in 24-well culture dishes at a density of 5×10^4 cells/0.5 ml of culture medium. For some experiments CBMCs were seeded onto Transwell membranes (0.4 μ m pore size; Nalge Nunc International, Naperville, IL) to separate them from the fibroblasts. CBMCs were maintained in co-culture for 4 days prior to any treatment; this interval was determined as optimal in preliminary kinetic experiments. Following this, co-cultures were incubated in 5% CSE for 48 h at 37°C in 5% CO₂. Cells were washed twice with Tyrode's buffer by gentle aspiration and then incubated with 10 μ g/ml of compound 48/80 for 30 min at 37 ° C in 5% CO₂. MC responsiveness was assessed by measuring the release of β -hex into culture supernatants as described above.

FLUORESCENCE MICROSCOPY

To evaluate the effects of CSE exposure on CBMC in suspension culture, cells at a density of 1×10^6 cells/ml were incubated for 48 h at 37 ° C in 5% CO₂ in medium containing 5% CSE. After washing with PBS, cytopspins were prepared for immunofluorescent staining and evaluation. To test the effect of CSE on interactions between MC and fibroblasts, fibroblasts were seeded onto 12-mm cover glasses and incubated at 37°C in 5% CO₂ until confluent. Then CBMCs (5×10^4 cells/0.5 ml) were seeded onto the fibroblast monolayers for 4 days. After this, the cells were maintained in co-culture for an additional 48h in medium with 5% CSE or in medium, alone (control). Immunofluorescence staining was performed directly using the co-cultured cells on the cover glasses. Both suspension-cultured and co-cultured cells were fixed for 10 min at room

temperature in 4% formaldehyde (Mallinkrodt Baker BV, Deventer, The Netherlands) in blocking buffer consisting of HBSS containing 0.1% (w/v) bovine serum albumin (BSA), permeabilized for 5 min at room temperature in blocking buffer containing 0.2% (v/v) Triton X100 (Merck, Darmstadt Germany), then incubated for a further 20 min in blocking buffer. Immunostaining was performed by incubating the cells first with rabbit anti-human Gi3 β (at a concentration of 5 μ g/ml in blocking buffer, from Santa Cruz Biotechnologies, Santa Cruz, CA) for 1 h at room temperature. After washing with PBS, slides were incubated with Cy5-conjugated anti-rabbit secondary antibody (obtained from Jackson ImmunoResearch Laboratories, West Grove, PA and diluted to 1:200 in blocking buffer) for 1 h at room temperature. Negative controls consisted of cell preparations that received only the second antibody. Slides were examined with Zeiss LSM 410 confocal laser scanning system attached to a Zeiss Axiovert 135 M inverted microscope equipped with a 63x/1.2 C-Apochromat water immersion lens (Carl Zeiss, Thornwood, NY).

INTRACELLULAR STAINING FOR FLOW CYTOMETRIC ANALYSIS.

CBMC were sensitized with 5 μ g/ml of human IgE as described above. The sensitized cells were subsequently incubated for various times (0, 1, 3, 15, 30 or 60 min) with both 5% CSE and anti-IgE. Cells were fixed after the indicated time points in 4% formaldehyde in blocking buffer for 15 min at room temperature, and then permeabilized in blocking buffer containing 0.1% saponin (Sigma-Aldrich), BSA (1mg/ml) and human AB serum (10%) for 30 min on ice. The cells were subsequently stained with one of the following primary antibodies: rabbit anti-phospho ERK1/2 (BioSource, Camarillo, CA), mouse anti-phospho P38 (BD Biosciences, Franklin Lakes, NY), or mouse anti-phospho JNK (Cell Signaling Technology, Beverly, MA). Parallel negative control cells were stained with the appropriate isotype control (either mouse IgG1 or rabbit IgG1, both from Dako, Glostrup, Denmark). All primary antibodies were used at a concentration of 1 μ g/ml (in blocking buffer) and were added to the fixed, permeabilized cells and incubated for 30 min on ice. The cells were subsequently incubated with either Cy5-conjugated anti-mouse or Cy5-conjugated anti-rabbit antibodies (both from Jackson ImmunoResearch Laboratories, and used at dilutions of 1:00 and 1:200, respectively), accordingly and incubated for 30 min on ice. Immunostained MCs were analyzed using a Becton Dickinson FACScalibur flow cytometer (Becton Dickinson,

San Jose, CA). For each staining at least ten thousand events were collected and data analysis was performed using CellQuest software (Becton Dickinson, Mansfield, MA).

STATISTICAL ANALYSIS

For data derived from viability and activation (β -hex release) experiments, mean values were compared by 2-way ANOVA. When the ANOVA probability value was significant [$p < 0.05$], the control and experimental groups were compared by t-test. The data are expressed as mean \pm standard error of the mean (SEM) of at least three independent experiments performed in triplicates. The Microsoft ExcelTM analysis tool-pack was used to perform the statistical analysis.

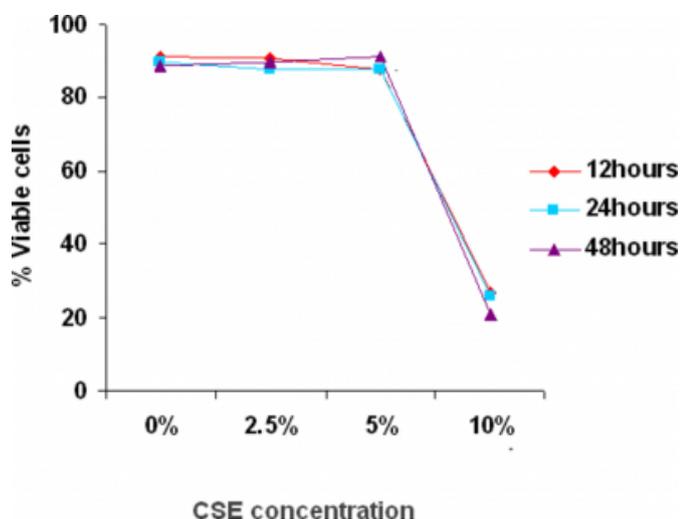
RESULTS

CSE CONCENTRATIONS HIGHER THAN 5% HAVE A TOXIC EFFECT ON HUMAN CBMCS

CBMCs (1×10^6 cells/ml) were cultured in medium alone or in the presence of different concentrations of CSE (2.5-10%) for 12, 24 or 48 h. Cell viability was assessed by Trypan blue exclusion. As shown in Fig. 1, cell viability did not change after incubating the cells with CSE at concentrations of 2.5 and 5% for 12, 24, and 48 h in comparison with cells treated with medium alone. However, exposure to 10% CSE significantly reduced CBMC viability from $91 \pm 2\%$ to $27 \pm 2\%$ ($p < 0.01$), from $90 \pm 1\%$ to $26 \pm 2.5\%$ ($p < 0.01$), and from $89 \pm 7\%$ to $21 \pm 2\%$ ($p < 0.01$), after exposures of 12, 24, and 48 h, respectively.

Figure 1

Figure 1: Effect of CSE on CBMC viability. CBMC were exposed to CSE at the concentrations indicated for 12, 24, and 48 h. Cell death was assessed by uptake of Trypan blue. Results are the mean \pm SEM of three independent experiments. $p < 0.01$ for all the comparisons.

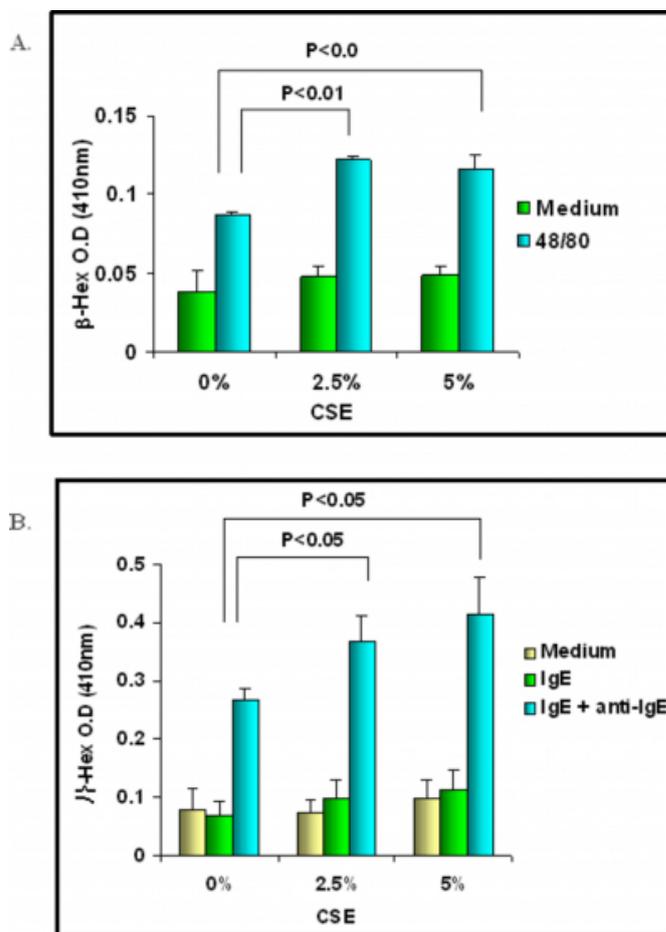


CSE ENHANCES IGE-INDEPENDENT AND IGE-DEPENDENT ACTIVATION OF CBMCS

To evaluate whether CSE might influence CBMC degranulation following IgE-independent activation, CBMC were cultured in medium alone, or in the presence of either 2.5% or 5% CSE for 48 h, then activated with compound 48/80 (5 μ g/ml) for 30 min. As shown in Fig. 2A, CSE at both concentrations significantly enhanced CBMC degranulation (as measured by β -hex release) following compound 48/80 activation in comparison with cells activated in medium without CSE ($p < 0.01$).

Figure 2

Figure 2: Effect of CSE on IgE-independent (A) and IgE-dependent (B) activation of CBMC. A. CBMC (10 cells/ml) were cultured in medium alone or in the presence of different concentrations of CSE (2.5-5%) for 48 h. The cells were subsequently activated with compound 48/80 (5 μ g/ml) for 30 min. B. Following sensitization of CBMC (10 cells/ml) with IgE (5 μ g/ml) for 5 days in culture medium, CSE (0-5%) was added and the cells were incubated for additional 48 h. Thereafter, the cells were activated with anti-IgE antibodies (10 μ g/ml) for 30 min. MC degranulation was measured by β -hex release assay. Values represent the mean \pm SEM. Results are the mean \pm SEM of three independent experiments.



To determine if exposure of CBMC to CSE also influences their degranulation following IgE-dependent activation, CBMC were sensitized with IgE (5 μ g/ml) in culture medium for 5 days. CSE was then added to final concentrations of 2.5% and 5% and the cells were incubated for an additional 48 h. The treated cells were subsequently activated by incubation with anti-IgE (10 μ g/ml) for 30 min. As shown in Fig. 2B, CSE at both concentrations also significantly enhanced CBMC degranulation after IgE-dependent activation in comparison with cells activated in

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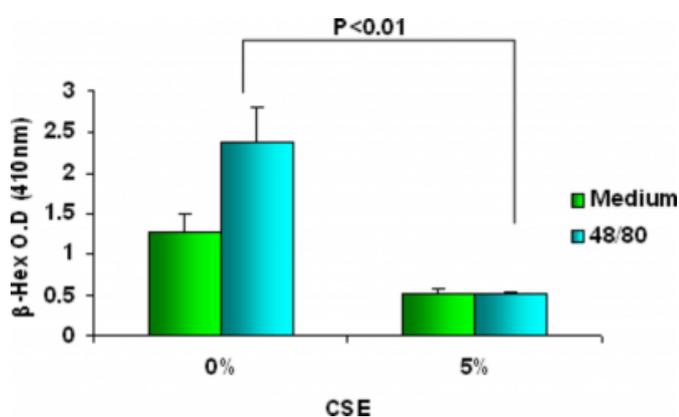
medium without CSE ($p < 0.05$). As shown in both parts of Fig. 2, CSE at all concentrations used did not affect the release of β -hex from CBMC that were not activated indicating that CSE does not directly induce degranulation.

CSE inhibits degranulation of CBMC co-cultured with 3T3 fibroblasts

MCs in peripheral tissues are surrounded by other cells such as fibroblasts. Thus it is believed that the MC response to stimuli is highly dependent upon their microenvironment. Hence, we determined the effect of CSE on the responsiveness of CBMC co-cultured with fibroblasts in an effort to mimic the MC microenvironment *in vivo*. CBMC were co-cultured with 3T3 fibroblasts in either the presence or absence of 5% CSE and subsequently activated with compound 48/80. As shown in Fig. 3, 5% CSE significantly inhibited degranulation of CBMC ($p < 0.01$) following compound 48/80 activation in comparison with cells activated in medium without CSE, as measured by β -hex release.

Figure 3

Figure 3: Effect of CSE on degranulation of CBMC co-cultured with 3T3 fibroblasts. CBMC (5×10^5 cells/0.5 ml) were maintained in co-culture with 3T3 fibroblasts for 4 days in medium alone before any treatment. Cells in the co-culture system were maintained for additional 48 h in medium alone or medium containing 5% CSE. The cells were then activated with compound 48/80 ($5 \mu\text{g/ml}$) for 30 min. Cell degranulation was measured by the release of β -hex. Results are the mean \pm SEM of three independent experiments performed in triplicates



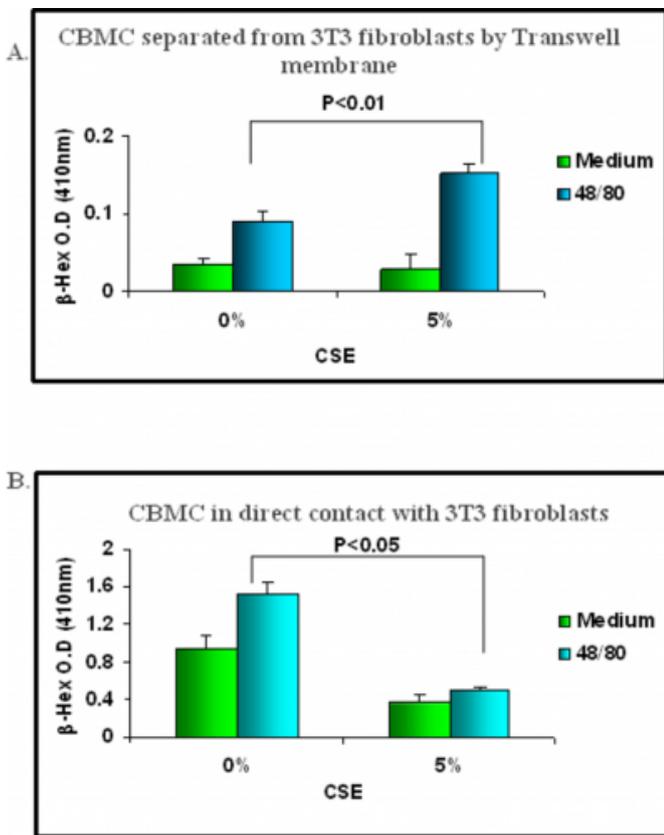
CSE inhibition of CBMC degranulation in 3T3 fibroblast co-culture is not dependent upon soluble fibroblast-derived factors

To assess whether soluble fibroblast-derived factors might be responsible for the inhibition of CBMC degranulation,

CBMC were cultured either directly on 3T3 fibroblast monolayers or separated from the fibroblasts using a Transwell membrane. In both treatments cells were cultured in medium alone for 4 days, and then maintained for additional 48h in medium alone (control), or in the presence of 5% CSE. The cells were activated with compound 48/80 ($5 \mu\text{g/ml}$) for 30 min. As shown in Fig. 4A, 5% CSE did not inhibit the degranulation of CBMCs that were separated from 3T3 fibroblasts by the Transwell membrane. In fact, in keeping with what was observed in suspension cultures (described above) CSE enhanced the response of CBMCs ($p < 0.01$) to compound 48/80 stimulation as evidenced by increased release of β -hex. However, as shown in Fig. 4B 5% CSE significantly inhibited the degranulation of CBMC that were in direct contact with fibroblasts ($p < 0.05$). This observation suggests that a fibroblast membrane-associated factor rather than a soluble one is responsible for inhibiting CBMC degranulation.

Figure 4

Figure 4: Effect of CSE on CBMC degranulation in fibroblast co-cultures. CBMC were maintained in the following conditions in the presence of soluble SCF (100 ng/mL) for 4 days before any treatment: (A) in direct co-culture with 3T3 fibroblasts, (B) in Transwell membrane to separate them from 3T3 fibroblasts. 5% CSE was added to test samples, while control samples received medium alone and cells were maintained for additional 48 h. Thereafter, all cells were activated with compound 48/80 (5 μ g/ml) for 30 min. Cell degranulation was measured by β^2 -hex release assay. Values represent the mean \pm SEM of 5. Note the scale differences on the Y-axis between (A) and (B), showing the much higher release of β^2 -hexosaminidase in CBMC that were in direct contact with fibroblasts.



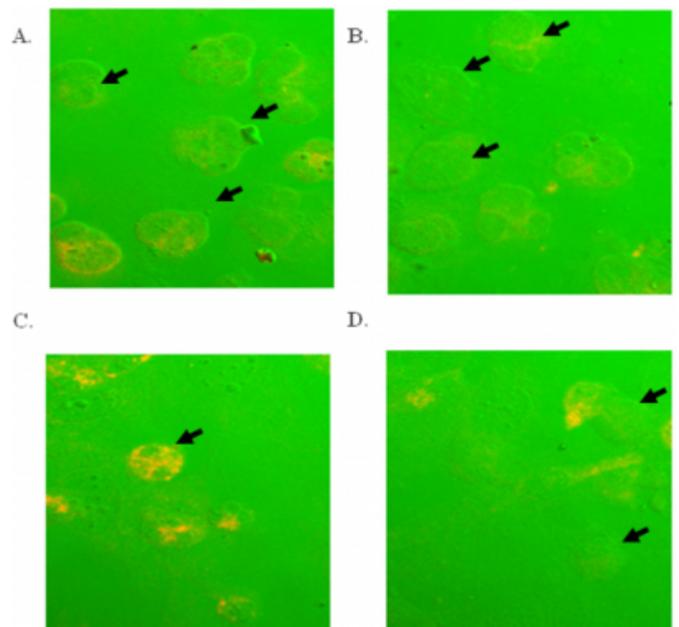
CSE ABROGATES THE EXPRESSION OF Gi3 IN CBMC

We also investigated a possible mechanism by which CSE might inhibit degranulation of CBMC that were in direct contact with fibroblasts. Other studies have shown that CBMC co-cultured with fibroblasts are more responsive to IgE-independent activation [44]. It is also known that IgE-independent activators stimulate rat peritoneal MC by directly activating Gi3, a pertussis toxin-sensitive Gi protein that leads to MC exocytosis and eicosanoid generation [11]. Piliponsky et al. [44] showed also that the increased

responsiveness of CBMC to IgE-independent activation was due to increased Gi3 protein expression following co-culture of CBMCs with 3T3 fibroblasts. Therefore, we investigated the expression of this protein following 48h incubation with 5% CSE in CBMC, either maintained in suspension, or in co-culture with 3T3 fibroblasts. Confocal microscopy images of Gi3 expression in CBMC are shown in Fig. 5. In CBMCs cultured in medium alone (Fig. 5A), Gi3 protein expression appeared higher than that of CBMC maintained in medium containing 5% CSE (Fig. 5B). Similarly, CBMC that were co-cultured with fibroblasts and maintained in medium alone, showed higher Gi3 expression (Fig. 5C) compared to CBMC that were cultured with fibroblasts and incubated with 5% CSE (Fig. 5D). Thus, exposure to 5% CSE appeared to reduce Gi3 protein expression.

Figure 5

Figure 5: Effect of CSE on the expression of Gi3 protein in CBMC. Confocal microscopy images of Gi3 protein (Cy5, orange fluorescence) showing: (A) Gi3 localization in CBMC maintained in medium alone for 6 days; (B) no expression in CBMC cultured in medium alone for 4 days and cultured for additional 48 h in medium containing 5% CSE; (C) Gi3 localization in CBMC co-cultured with 3T3 fibroblasts for 6 days; (D) no expression in CBMC co-cultured with 3T3 fibroblasts for 4 days and maintained in medium containing 5% CSE for additional 48 h. The displayed figures are from a representative experiment of two. Magnification: Fig. 10A-10D, 63x.



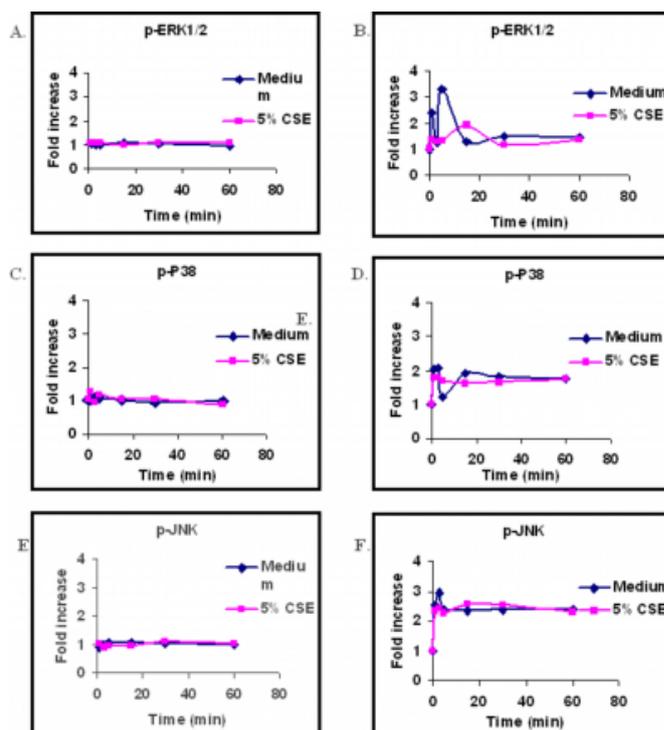
Exposure to CSE influences the kinetics of MAPK phosphorylation in CBMC following IgE dependent

activation

To determine whether CSE influences MC activation by affecting the three major MAP kinases (ERK1/2, P38, and JNK) we performed the following experiments in which CBMCs were activated through the IgE-dependent pathway. CBMCs that were previously sensitized with IgE in culture medium for 5 days, were incubated for various times (0-60 min) with 5% CSE and/or anti-IgE. Phosphorylated kinase levels were assessed by intracellular staining with specific antibodies and quantified by flow cytometry. As shown in Figs. 6A, C, and E exposure of unactivated CBMCs to CSE did not affect the phosphorylation of all three MAPKs, in comparison to what was observed in activated cells (Fig. 6B, D, and F). All comparisons were made to cells maintained in medium without CSE. ERK1/2 phosphorylation in unexposed CBMC was biphasic, consisting of two peaks that were detectable at 1 and 5 min after activation. Phosphorylation was markedly decreased in the presence of 5% CSE. In activated CBMCs, exposure to 5% CSE altered the kinetics of ERK1/2 phosphorylation causing a peak only at 15 min after activation. In contrast, ERK1/2 phosphorylation in CBMCs cultured in medium occurred earlier and with greater magnitude (Fig. 6B). CSE concentrations of 5% also reduced P38 phosphorylation occurring in activated CBMCs between 1-3 min after activation; however, CSE also inhibited de-phosphorylation that was observed after 5 min (Fig. 6D). CSE also reduced JNK phosphorylation that peaked in activated CBMCs between 1-5 min. However, exposure to 5% CSE appeared to induce late JNK phosphorylation between 5-30 min (Fig. 6F).

Figure 6

Figure 6: Effect of CSE on the kinetics of MAPK phosphorylation in CBMC following IgE-dependent activation. CBMC Intracellular FACS staining of MAPKs. CBMCs were sensitized with 5 μ g/ml of human IgE for 5 days. A, C, and E: CSE (5%) was added to test samples for the indicated time points. B, D, and F: CBMC were activated for the indicated time points with anti-IgE alone or together with 5% CSE. Rabbit anti-phospho-ERK1/2, mouse anti-phospho-P38, or mouse anti-phospho-JNK antibodies (1 μ g/ml) were added to the fixed and permeabilized cells (30 min on ice) and then incubated with Cy5 conjugated anti-mouse or Cy5 conjugated anti-rabbit antibodies (1:100 and 1:200 respectively, 30 min on ice). Cells were analyzed on a Becton Dickinson FACS-Calibur System (Becton Dickinson, San Jose, CA). For each staining at least ten thousand events were collected and data analysis was performed using CellQuest software (Becton Dickinson, Mansfield, MA). The data shown are represented by fold increase/baseline time 0 and are from one typical independent experiment out of 3.



DISCUSSION

MCs reside in barrier tissues, including the skin and the mucosa of the respiratory and gastrointestinal tracts where they are exposed to numerous environmental stimuli. Since MC activation results in the coordinated release of pro-inflammatory mediators into the surrounding tissue, inappropriate activation of this cell type following exposure to environmental challenges may result in chronic inflammation. MC activation has been proposed to

contribute to pathologies that characterize the airways of tobacco smokers, including: lung cancer, chronic obstructive pulmonary disease (COPD); emphysema; and exacerbation of asthma [33, 44, 45, 46, 47, 48, 49].

Several clinical studies have shown that actively smoking asthmatics have more severe symptoms, accelerated lung function decline, and increased morbidity and mortality in comparison to non-smoking asthmatics [26]. There is also strong evidence that secondary exposure to tobacco smoke adversely affects the health of children with asthma [24]. The potential role of CS exposure in the pathogenesis was revealed by Oryszczyn, et al. [27] who observed that asthmatic smokers had significantly higher IgE levels than non-smokers. Another studies reported that CS dose-dependently increased the numbers of neutrophils and macrophages in the bronchoalveolar lavage fluid (BALF) of healthy adult smokers compared with those of non-smokers and was also associated with altered airway cytokine regulation [28], the ability of the airway epithelium to support repair [29] and clinical responses to corticosteroid therapy [30]. Kalenderian et al. [31] observed that the levels of the MC mediators, histamine and tryptase, were considerably elevated in BALF from smokers compared to nonsmoker indicating “a greater propensity for MC-mediated injury in the smoker” which was borne out by subsequent studies [32, 33]. Thomas et al. [50] reported that a water-soluble extract (both gas and particulate phases) of CS dose-dependently increased the release of histamine and tryptase from a canine mastocytoma cell line. Histamine release in response to the calcium ionophore calcimycin (A23187) was not affected by the extract suggesting that a Ca^{2+} -independent mechanism was involved. Small-Howard et al. [51] showed that exposure of MC to a CS condensate exposure increased proteinase expression at both the mRNA and protein levels. In contrast, Flower et al. [52] reported that a water-soluble constituent of CS reduced the ability of rat basophilic leukemia 2H3 (RBL-2H3) cells to degranulate in response to both immunological and non-immunological stimuli which included compound 48/80, calcimycin, substance P, concanavalin A and antigen. However the mechanism(s) by which CS affects MC function were not investigated in these studies. Here, we investigated, using in vitro techniques, the effects of CSE upon MC survival and activation using CBMC as a study model. As earlier studies involved MC of animal origin [51, 52] we decided to investigate CS effects (as a soluble extract) on human MC properties in vitro. CSE enhanced β -hex release after compound 48/80 activation in

human CBMCs. This was in contrast to what we had observed earlier in rat peritoneal MC (data not shown). The β -hex response to IgE-dependent activation was also enhanced in CBMCs following exposure to CSE. While the effect of CSE on MC's appears to be dependent on their species origin and tissue source, our observations were compatible with what has been observed clinically in smokers with lung disease.

In tissues, MC's interact with other cells such as fibroblasts. These interactions mediate survival, growth regulatory, and activation signals [53]. Piliponsky et al [44], observed that CBMC and human lung-derived MCs were minimally responsive to eosinophil-derived major basic protein (MBP) and compound 48/80 when challenged in suspension. However, when cultured with 3T3 fibroblasts, MBP-stimulated CBMC became more responsive and released more histamine and PGD_2 , suggesting that cellular cross talk is required for optimal MC activation. In this study we observed that for CBMC co-cultured with fibroblasts the β -hex response to compound 48/80 activation was markedly reduced in the presence of CSE. In fact, exposure to CSE reduced the basal release of β -hex to a level that might be comparable to that detected in unactivated MC maintained in suspension culture. Thus, while exposure to CSE appeared to augment β -hex release of activated CBMCs in suspension culture, quite the opposite effect was observed when fibroblasts were present. We also observed no difference in the net increase in β -hex release from activated CBMCs separated from 3T3 fibroblasts by a Transwell membrane and CBMCs maintained alone in suspension. This observation suggests that soluble mediators are unlikely to be involved and that the observed inhibitory effect of CSE required cell contact.

The membrane form of SCF in fibroblasts appears to be responsible for up-regulating the expression of the β -subunit of Gi3, a protein that regulates exocytosis in MC [11]. Up-regulation of Gi3 β expression may contribute to MC responsiveness to IgE-independent activators [44]. To better define the effect of CSE on MCs, we investigated its effect on Gi3 β expression in CBMCs. We observed that CSE dramatically inhibited expression of Gi3 β in CBMCs maintained in suspension. This finding is at odds with the augmentation of β -hex release by CSE of CBMCs maintained in suspension culture. CSE also inhibited increase in Gi3 β expression when the cells were co-cultured with fibroblasts, suggesting a possible interaction of CSE components with

the membrane form of SCF expressed on fibroblasts. Alternatively, CSE may interact with the SCF receptor c-Kit, a member of type III receptor tyrosine kinase family found on MC. Whether CSE affects membrane SCF found on fibroblasts, or its receptor c-Kit on MC, or both, requires further investigation.

Stimulation of high affinity IgE Fc receptors (Fc ϵ RI) on MCs activates the tyrosine kinases, Lyn and Syk, and causes tyrosine phosphorylation of phospholipase C- β [53], resulting in the Ca²⁺ and protein kinase C-dependent secretion of inflammatory mediators [54, 55]. Concomitantly, Fc ϵ RI stimulation initiates signaling events resulting in the activation of MAPKs that in turn, regulate nuclear responses, including cytokine gene expression [56]. To investigate whether CSE influenced the phosphorylation of different MAPKs following IgE-dependent activation, CBMC were incubated with 5% CSE and anti-IgE following pre-sensitization of the cells with IgE, and phosphorylated ERK1/2, p38, and JNK levels were assessed by intracellular staining with specific antibodies. CSE had no direct effect on the phosphorylation of any of these activation pathways. However, when the cells were immunologically activated, CSE exposure decreased phosphorylation of ERK1/2, p38, and JNK, suggesting that CSE interferes with MAPK signalling in MCs. Whether the observed decreased phosphorylation of ERK1/2, p38, and JNK influenced the functional properties of CBMCs such as the production of cytokines or other mediators was not investigated.

Characterization of the components of the CSE used herein and the physiologic relevance of their concentrations was beyond the scope of the present study. However, CS is a complex medium containing approximately 4000 different constituents [57] separated into gaseous and particulate phases. The components of the gaseous phase include carbon monoxide, carbon dioxide, ammonia, hydrogen dioxide, hydrogen cyanide, volatile sulphur-containing compounds, nitrogen oxides (including nitric oxide, NO), and other nitrogen-containing compounds. The particulate phase contains nicotine, water and tar [57]. Since CS contains high levels of NO, solubilized NO in CSE might be a prime candidate affecting MC responsiveness. Xiu, et al [58], showed that the addition of NO inhibitors and scavengers did not, however, reverse the effect of CSE in RBL-2H3 cell line. However, adding the NO donor (SNP) did not mimic the effects of CSE either. In the presence of the free radical scavenger, N-acetyl-cysteine CSE inhibition of β -hex release

was partially reversed, suggesting that CSE may exert its effects on MC via oxidative free radicals. Flower, et al [52] used two different CS extracts from standard and low-tar cigarettes and concluded that the effects of CSE could not be attributed to the pharmacological activity of nicotine. This is consistent with the report of both Thomas, et al. [50] who found that nicotine tartrate had no effect upon the basal release of histamine, or upon calcimycin- induced PGD₂ release from canine mastocytoma cells, and that of Xiu, et al [57], who also found that nicotine did not mimic the effects of CSE. Since these studies were carried out in animal cell lines, the effects of these particular CS constituents on human MC function remain to be elucidated.

In conclusion, we demonstrated that in vitro exposure of human MC to soluble components of CS impacts significantly on their survival, activation, and interactions with stromal cells such as fibroblasts. Decreased phosphorylation of ERK1/2, p38, and JNK that was observed following exposure of the cells to CSE suggests that CSE interferes with MAPK signalling in MCs. The observed decrease in the level of Gi3 β protein when CBMCs were exposed to CSE suggests a possible mechanism by which CS decreases CBMC degranulation. While our observations suggest that CSE impairs CBMC activation through its inhibition of Gi3 β and interference with MAPK signaling, the observed effect of CSE in augmenting β -hex release in activated CBMC maintained in suspension culture is incongruous and requires further investigation. Moreover, our findings suggest that exposure of human MC to the soluble components of CS may not only modulate their functional properties directly but also indirectly through their interactions with stromal cells in the lung microenvironment. Thus, through its effects on MC, CS has the potential to influence the severity and course of lung diseases.

ACKNOWLEDGEMENTS

This study was supported by funds from the Aimwell Charitable Trust (UK). The authors declare that, to their knowledge, there are no conflicts of interest.

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