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Inflammation Research

Induced sputum of patients with chronic obstructive pulmonary disease (COPD) contains adhesion-promoting, therapy-sensitive factors

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Abstract. *Objective:* The aim of this study was to investigate whether sputum of COPD patients before and after treatment with inhaled corticosteroids (IHC) or N-acetylcysteine (NAC) exerts any effect on the adhesion of isolated polymorphonuclear cells (PMNs) to cultured endothelial cells.
Methods: A human endothelial cell line was grown to confluence before use in adhesion experiments. PMNs were obtained from normal, non-smoking volunteers and preincubated (30 min, 37°C) with diluted sputum sol obtained from COPD patients before the cells were put on the endothelial cells.
Results: Basal adhesion of unstimulated PMNs after 30 min at 37°C in 5% CO_2 was 15.9 ± 1.1% (mean ± SEM, n = 9). A significant enhancement of the adhesion to 33.0 ± 1.4% (n = 11, P < 0.0001) was observed with sputum obtained from COPD patients before treatment with IHC, and 34.6 ± 1.5% (n = 10, P < 0.0001) before treatment with NAC. Administration of IHC for 8 weeks resulted in an adhesion of 27.7 ± 2.4%, which is an inhibition of 31% (n = 11, P < 0.05). However, treatment for 8 weeks with NAC showed no change in the adhesion of stimulated PMNs. Long-term treatment with NAC showed a gradual decrease of adhesion (n = 9, P < 0.05), whereas long-term treatment with IHC lead to an increase in adhesion (n = 10, P < 0.02).
Conclusions: These results indicate that factors locally produced in the airways of COPD patients may promote adhesion of neutrophils to endothelium. They further suggest that glucocorticoids may only have a short-term transient effect on adhesion, whereas NAC showed effects on the adhesion after administration for longer periods.

Key words: COPD – Adhesion – Induced sputum – Granulocytes – Endothelial cells

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Introduction

Damage to lung tissue as a result of an inflammatory response is usually accompanied by an influx of neutrophilic granulocytes. In patients with chronic obstructive pulmonary disease (COPD) an enhanced number of neutrophils was found both in the airways and in bronchoalveolar lavage fluid (BAL) [1–3]. The initial step in this process is the expression of adhesion molecules by vascular endothelial cells in the vicinity of the inflammatory spot, followed by an interaction between adhesion molecules of both endothelial cells and neutrophils. Next step is the action of infiltrating neutrophils upon other cells in the lung, with release of toxic metabolites such as oxygen radicals and proteolytic enzymes [3, 4] leading to extension of inflammation [5, 6]. The intensity of inflammation may be reduced by use of anti-inflammatory drugs [7]. In a model of extracorporeal circulation during cardiac surgery with activation of neutrophils and complement, it was shown that administration of either corticosteroids or the anti-oxidant N-acetylcysteine (NAC) could inhibit the production of proinflammatory cytokines both in peripheral blood and in the lung [6, 8].

So far, few reports on adhesion molecules in COPD have been published [9–13]. The same may be true for possible effects of drugs on adhesion mechanisms in COPD. It was reported that soluble intercellular adhesion molecule (ICAM-1) in both serum and BAL fluid of COPD patients was elevated in comparison with normal individuals, although in serum there was some overlap with normal values [10]. Recently, elevated levels of cell-bound ICAM-1 were reported [11, 12]. High concentrations of the cytokines interleukin-8 (IL-8) and tumour necrosis factor-α (TNF-α) in induced sputum from patients with COPD have been reported [14] and these cytokines may be involved in the recruitment of neutrophils [15, 16]. The role of corticosteroids in the management of COPD is controversial [17, 18]. Cytokine gene expression is known to be reduced both in vitro [19] and in vivo [20] in response to corticosteroids, and this has been postulated as a mechanism of action of

corticosteroids in this condition [21]. If steroids do play a role in the treatment of COPD, it is reasonable to suggest that they act via down-regulation of cytokines and adhesion molecules. The latter may also be true for the reducing effect of NAC on cell migration and activation [8, 22, 23].

The purpose of the present study was to evaluate the capability of induced sputum from patients with COPD to promote adhesion during a long-term period of therapy with inhaled corticosteroids (IHC) or NAC. Therefore, we analysed the interaction of sputum-stimulated granulocytes with immortalised human endothelial cells.

Materials and methods

Reagents

Isotonic shock solution contained 155 mM NH_4Cl, 10 mM $KHCO_3$, 0.1 mM ethylenediaminetetra-acetic acid, disodium salt (EDTA) and 10 mg/L of phenolred. The pH of the solution was adjusted to 7.40 at 0°C.

Tissue culture media, glutamine, foetal calf serum, trypsin/EDTA solution, Hanks' Balanced Salt Solution (HBSS) and Dulbecco's Phosphate Buffered Saline (PBS) were purchased from Life Technologies, Paisley, UK; Ficoll-Hypaque (Histopaque 1077), EDTA, phenolred, bovine serum albumin (BSA) and lipopolysaccharide from *Escherichia coli*, serotype O111:B4 were obtained from Sigma Chemical Co., St. Louis, MO, USA; calcein-AM was purchased from Molecular Probes Europe, Leiden, The Netherlands and ECV-304, human endothelial cell line was obtained from the European Collection of Animal Cell Cultures, Salisbury, UK. All other chemicals used were reagent grade and obtained from Merck, Darmstadt, Germany.

Patient selection and sputum induction

Patients with COPD were considered to be clinically stable (no exacerbation). The inclusion criteria for these patients were smoker or ex-smoker, irreversible airway obstruction (≤10% of baseline), FEV_1 is ≤80%, but ≥50% and absolute 1.5 litre, FEV_1/VC is ≤80% and there should be no history of atopy or asthma. These patients started with a wash-out period of 8 weeks during which β_2-agonists were only allowed when necessary. A baseline was obtained after this first period. During the subsequent treatment period the patients were treated with either inhaled budesonide (800 µg, twice daily), or oral NAC (600 mg, once a day) for a period of 10 months. Follow-up was at 2, 4, 6 and 10 months. The study was performed in a randomised, double-blind cross-over fashion. At the start of each new period and at follow-ups, sputum was collected and handled as described earlier [24]. Sputum sol phase was used for incubation with isolated granulocytes. As a positive control, neutrophils were stimulated with 100 ng/ml lipopolysaccharide (LPS), *Escherichia coli*, serotype O111:B4.

Isolation of human granulocytes

Granulocytes were isolated from heparinised venous blood of healthy, non-smoking volunteers, with no known history of pulmonary disease. Granulocytes were separated from mononuclear cells by centrifugation on Ficoll-Paque, 1.077 g/ml for 25 min, 1000 × g at room temperature. Contaminating erythrocytes were removed from the granulocyte suspension by isotonic ammoniumchloride lysis at 0°C [25] and subsequent centrifugation. The granulocytes were collected, washed twice in PBS and suspended in PBS at a concentration of 10^7/ml.

Loading of granulocytes with fluorescent dye

To each millilitre of granulocyte suspension a volume of 5 µl calcein-acetomethylester (calcein-AM, 5 µM in DMSO) was added, followed by incubation at 37°C for 15 min. After labelling was stopped by adding HBSS, not incorporated label was removed by washing the cells twice in HBSS (400 × g, for 10 min at room temperature). Afterwards the cells were resuspended in HBSS/0.01% BSA at 2×10^6 cells/ml and incubated in humidified air with 5% CO_2 for 1 h at 37°C to activate the label.

Adhesion assay

The human endothelial cell line ECV-304 was grown to confluence in M199, supplemented with 2 mM glutamine, 10% foetal calf serum and 1% penicillin and streptomycin solution (100 U/ml, 10 µg/ml respectively) [26]. Cells were cultured at a density of $2 \times 10^4/cm^2$ in uncoated culture flasks (Falcon, 75 cm^2, Becton Dickinson) in humidified air with 5% CO_2 at 37°C for 3–5 days until confluence was observed. At confluence the cells were detached using 0.05% trypsin/EDTA in Modified Puck's Saline A. From the 2nd to the 5th passage endothelial cells were transferred into 24 well cell cluster plates (Costar) at a concentration of 4×10^4 cells per well to form a monolayer. After 2 days confluent monolayers were obtained and used in adhesion experiments. Immediately before the assay, the integrity of the monolayer was microscopically controlled, and cells were washed three times with pre-warmed HBSS. The isolated and calcein-AM loaded neutrophils were pre-treated with or without sputum supernatant (sol fraction) for 30 min at 37°C. The sputum sol fraction was used in a final dilution of 1:100. After washing, to each well 250 ml of the pre-treated and calcein labelled granulocytes (2.5×10^5 cells) in HBSS were added and incubated with the endothelial cells without agitating for 30 min at 37°C. After this incubation, total fluorescence (F_t) was measured in a Cytofluor 2300 fluorometer (Millipore, Bedford, MA, USA) with an excitation wavelength of 485 nm and emission at 530 nm. Non-adherent cells were removed by gentle washing with HBSS, followed by detection of fluorescence to assess the amount of adhering cells (F_x). The percentage of adherent leukocytes was calculated using the formula: % adhesion = $(F_x - \text{blank}/F_t - \text{blank}) \times 100$, where blank is the fluorescence of 250 ml HBSS/0.01% BSA. The experiments were performed in quadruplicate for each experimental condition.

Statistical methods

All data are presented as mean ± standard error of the mean (SEM). Statistical analysis was performed using Student's t-test for comparisons between the baselines and the outcome of treatment for 8 weeks.

Results

The number of neutrophils in the induced sputum was counted. At baseline 30.1 ± 0.9% neutrophils were present. The number did not change significantly due to treatment with either IHC (36.1 ± 3.4%) or NAC (35.4 ± 3.9%). Total cell numbers were $6.7 \pm 2.3 \times 10^6$/g sputum at baseline and $3.5 \pm 1.0 \times 10^6$/g after IHC and $5.8 \pm 2.3 \times 10^6$/g after NAC, and hence not significant different. No specimens were excluded for excessive salivary contamination. Basal adhesion of neutrophils was between 4.6 and 29.8% with a mean of 15.9 ± 1.1% (n = 9). Stimulation of neutrophils with 100 ng/ml LPS increased their adhesion to 33.6 ± 1.7% (range: 16.1 – 54.0; n = 9, $P < 0.0001$). For neutrophils in-

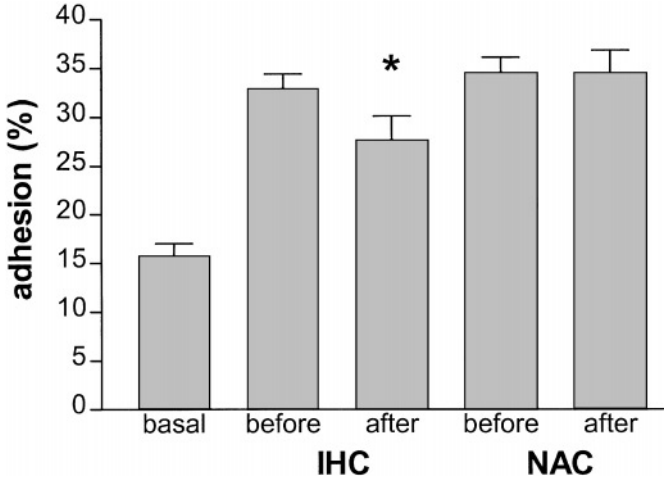

Fig. 1. Adhesion (%) of PMNs, unstimulated (basal) or stimulated with sputum sol of COPD patients at baseline and after 8 week treatment with either IHC or NAC. Results are expressed as mean ± SEM. * $P < 0.05$ as compared to adhesion before treatment.

cubated with the sputum sol phase obtained from non-treated COPD patients an increased adhesion of 33.0 ± 1.4% before treatment with IHC ($n = 11$, $P < 0.0001$), and 34.6 ± 1.5% before treatment with NAC ($n = 10$, $P < 0.0001$) was observed. When patients were treated with IHC during 8 weeks, sputum stimulation of neutrophil adhesion dropped to 27.7 ± 2.4% ($n = 11$, $P < 0.05$) (Fig. 1). This is a net inhibition of 31% as compared to the stimulatory effect of sputum before treatment and corrected for basal adhesion. In the same figure it is shown that treatment with NAC has no inhibiting or stimulating effects (34.6 ± 2.2%, $n = 10$, $P = 1.00$) on adhesion as compared to adhesion induced with sputum obtained before treatment. However, when a treatment period with NAC was extended to 10 months a gradual significant decrease in neutrophil adhesion was observed with a maximal inhibition of 40% ($n = 9$, $P < 0.05$) (Fig. 2B). On the other hand, the inhibiting effect of IHC was only found after 2 months of treatment. Prolonged administration of IHC led to a significant increase in adhesion ($n = 10$, $P < 0.02$) (Fig. 2A).

Discussion

Our results showed that sputum sol phase from COPD patients induced before treatment, is able to induce PMN adhesion to ECV-304 monolayers in culture. Administration of IHC during 8 weeks resulted in a lower adhesion, but this inhibition of adhesion disappeared very quickly during a prolonged treatment with IHC. However, treatment during 8 weeks with NAC had no inhibiting effect on the adhesion, but increasing the treatment period up to 10 months showed a gradual decrease of the adhesion of PMNs.

As a control, sputum from normal individuals was tested for its effect on neutrophil adhesion. Although it is quite difficult to obtain a good sample of induced sputum from normal non-smoking individuals, we managed to get some small samples. This normal sputum showed no adhesion promoting effect on the cells in our model.

We selected an endothelial cell line (ECV-304) as a representative for human endothelial cells in our studies to have a stable experimental model. In culture, ECV-304 cells express ICAM-1 and other adhesion molecules without stimulation [26]. No enhanced adhesion of unstimulated PMNs was observed when the monolayer was pre-incubated with 10 ng/ml IFN-γ or 10 ng/ml TNF-α as compared to unstimulated endothelial cells (data not shown). Since adhesion of PMNs to unstimulated endothelial cells could be measured in a sufficient and reproducible way, we decided not to "pre-stimulate" the endothelial cells.

A number of techniques have been described for quantifying cell adhesion to endothelial cells. Because of the many advantages, as easy performance, rapid, highly sensitive, and reproducible with the possibility of microscopic control, we preferred a fluorometric method for measuring granulocyte adhesion to endothelial cells [27, 28].

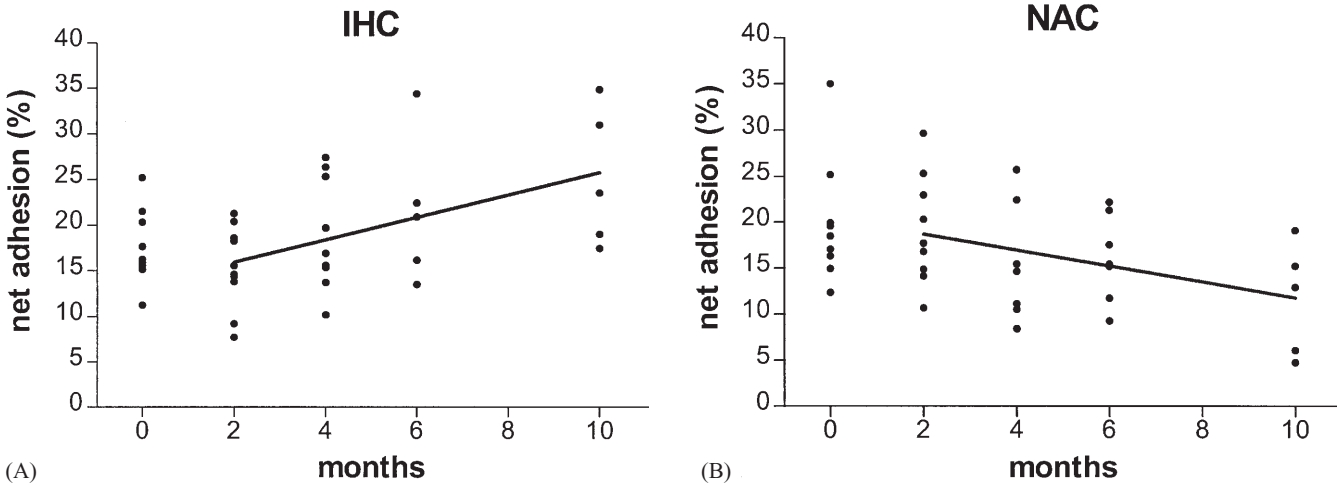

Fig. 2. (A) Effect of long-term treatment of COPD patients with IHC on the net adhesion (%) of stimulated PMNs to ECV-304 in response to induced sputum sol obtained from these patients ($r = 0.51$, $P = 0.004$). (B) Effect of long-term treatment of COPD patients with NAC on the net adhesion (%) of stimulated PMNs to ECV-304 in response to induced sputum sol obtained from these patients ($r = 0.41$, $P = 0.03$).

PMN adhesion plays a key role in their migration from blood vessels to the surrounding tissue [3] and one or more factors in the sputum may therefore contribute to this process by increasing PMN adhesion. Others have shown a lack of complete inhibition of neutrophil chemotactic activity in IL-1α stimulated endothelial cell supernatant by anti-IL-1α and anti-IL-8, suggesting that more than one adhesion promoting factor has been produced [29].

During the wash-out period, patients were allowed to use β_2-adrenergic receptor agonists such as bronchodilators, if necessary. cAMP enhancement in endothelial cells by β_2-adrenergic receptor agonists may attenuate the adhesion and chemotaxis of leukocytes [30, 31]. It was reported that pre-incubation of neutrophils with salmeterol inhibited the adhesion of stimulated neutrophils concentration dependently, and the level required for inhibition of adhesive interactions seemed to be dependent on the activation state of the neutrophil [32]. Moreover, it was shown that the inhibiting effects of cAMP elevating agents were even dependent on the chemoattractant used [33]. The presence of β_2-agonists in sputum was not investigated. If there was an effect of these drugs on the adhesion, one should expect it to work predominantly at the baseline levels. Since we found significant enhanced baseline values, we think the effect of this kind of drugs in this study might be neglected, but not be excluded.

Treatment of COPD patients for 8 weeks with IHC led to a decrease of PMN adhesion. However, prolonged treatment of the same patients up to 10 months with this type of steroid showed that the effect on adhesion was lost. In contrast to studies of steroid use in asthma, studies on the effects of steroids in COPD are scarce and usually showed no or small effects [34–37]. In these studies, the lack of effect may be due to the 2–3 weeks of administration of steroids, which is a relative short period. Only in one study a positive effect, selective increase in the protease inhibitor alpha-1 chymotrypsin, has been reported after 7 days of treatment with prednisolone [38]. On the other hand it may be that different steroids exert different working spectra [39]. So far, we do not have an explanation for the disappearance of the steroid effect, but it may be a result of glucocorticoid receptor downregulation by steroids [40] or it may be due to desensitisation of the receptor in the presence of high concentrations of agonist [41]. However, it is not certain whether this persists after prolonged treatment. Another point to focus on is the induction of transciption of adhesion molecules that may be the result of several different mechanisms. In that case it may be that steroids, or some of them, are not able to block the induction of adhesion molecules [39, 42].

NAC has been shown to reduce endotoxin-induced neutrophil activation in sheep [43] and to diminish neutrophilic lung inflammation [8, 22, 23]. We demonstrated that prolonged treatment with NAC might finally reduce the adhesion of PMNs to endothelial cells. In our model, NAC probably reduces adhesion by diminishing nuclear factor-κB (NF-κB) dependent gene transcription of adhesion molecules through a redox-sensitive mechanism [44, 45].

The patients used in this study were clinically stable, and had no exacerbations. The possibility that NAC treatment influenced the bacterial presence in the airways was not examined, but should be considered as one of the possibilities to lower PMN activation status and lowering of IL-8 levels [46, 47]. We also confirmed the presence of high levels of IL-8 in the sputum of COPD patients (data not shown). It was reported that IL-8 levels are elevated in both sputum and lavage fluid from COPD patients as compared to normal individuals [48, 49]. NAC treatment is able to reduce IL-8 levels in plasma [8], but in the present study no such effect was significantly observed in sputum (data not shown).

We have demonstrated that long-term treatment with IHC in patients with COPD has no inhibiting effect on the adhesion process of PMNs to endothelial cells by factors present in induced sputum of these patients. However, an inhibiting effect was only observed after shortterm treatment with IHC. This observation supports the clinical impression that these drugs may be of little value in COPD. The use of NAC in the treatment of COPD seems to be more promising, especially after longer periods (>6 months) of administration. The possible way of action through the blocking of NF-κB may affect the production of a wide variety of mediators and regulate inflammation much more profoundly, but it needs to be established yet.

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