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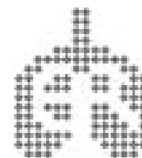

Airway neutrophilia in COPD is not associated with increased neutrophil survival

P. Rytilä*,#, M. Plataki*, F. Bucchieri*, M. Uddin*, G. Nong*, V.L. Kinnula¶ and R. Djukanović*

ABSTRACT: **Neutrophilic airway inflammation is a prominent feature of chronic obstructive pulmonary disease (COPD) and correlates with disease severity. The mechanisms that determine the extent of neutrophilia could involve increased influx or prolonged survival of neutrophils. The aim of the study was to assess whether neutrophil pro-survival mechanisms are increased in the airways of subjects with COPD owing to the presence of anti-apoptotic factors in the bronchial lining fluid.**

Induced sputum samples were collected from 20 subjects with stable COPD, 14 healthy smokers and 14 healthy controls. Quantification of apoptotic neutrophils was based on typical morphological cell changes. Anti-apoptotic, pro-survival activity in the sputum was studied by culturing peripheral blood neutrophils with the fluid phase of induced sputum. Apoptosis was assessed both by morphology and flow cytometry using Annexin V/7-aminoactinomycin D staining.

COPD patients and healthy smokers had significantly higher percentages of sputum neutrophils than healthy controls. However, there were no significant differences between the three subject groups in either the proportion of apoptotic neutrophils in sputum or the *in vitro* anti-apoptotic activity detected in the sputum fluid phase.

In conclusion, prolonged survival of neutrophils in sputum is not a feature of chronic obstructive pulmonary disease and cannot explain the increased numbers of airway neutrophils in this disease.

KEYWORDS: **Apoptosis, chronic obstructive pulmonary disease, inflammation, neutrophils**

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Neutrophil accumulation in the airways is recognised as a prominent feature of chronic obstructive lung disease (COPD), with the extent of neutrophilic infiltration in both the airway lumen and tissues correlating with disease severity [1–4]. It has been widely hypothesised that neutrophils become activated in the airways to release reactive oxygen species, proteases, and inflammatory cytokines, which play an important role in lung injury [5, 6]. However, little is known about the cellular and molecular mechanisms that control neutrophil migration and survival in the airways in COPD.

Neutrophil accumulation is a dynamic process that consists of the recruitment of neutrophils from the bloodstream and clearance from the lungs as a result of phagocytosis of apoptotic cells. Neutrophils are attracted from the vascular space into the airway lumen by chemotactic factors, such as interleukin (IL)-8, leukotriene (LT)B_4 and tumour necrosis factor-α. Previous studies have suggested that circulating neutrophils isolated from subjects with COPD are more responsive than neutrophils from healthy control subjects to chemotactic stimuli [7]. However, it is not known whether prolonged cell survival due to reduced apoptosis of neutrophils in the airways also contributes to the accumulation of these cells.

Apoptosis is a morphologically and biochemically distinct form of cell death that limits tissue injury and is an essential requirement for the resolution of inflammation [8]. Neutrophil apoptosis has been studied extensively and shown to involve typical morphological changes, attenuation of activation and degranulation, nuclear and cytoplasmic condensation, DNA fragmentation, dilatation of the endoplasmic reticulum and alterations in cell membrane composition.

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Further characteristic features are activation of caspases and leakage of cytochrome *c* from the mitochondria [9, 10]. Apoptotic neutrophils are removed by macrophages using phagocyte recognition mechanisms [8]. Dysregulation of neutrophil apoptosis leads to the accumulation of neutrophils within the tissue, persistence of inflammation and increased potential for tissue damage. A variety of pro-inflammatory cytokines and other mediators have been demonstrated to delay neutrophil apoptosis *in vitro*; these include granulocyte macrophage-colony stimulating factor (GM-CSF), IL-8, and LTB_4, all of which have been shown to be increased in the airways of subjects with COPD [11].

Little is known about the role of neutrophil apoptosis in COPD. Two studies that examined the spontaneous apoptosis of peripheral blood neutrophils [12, 13] found no difference in the rate of *in vitro* apoptosis of circulating neutrophils from subjects with stable COPD when compared with healthy smokers or healthy control subjects. The present authors have hypothesised that the presence of elevated concentrations of pro-neutrophil factors in the airways prolongs neutrophil survival, resulting in fewer apoptotic neutrophils being seen in the sputum of subjects with COPD. To test this hypothesis, induced sputum, a medium that samples the airway lining fluid and is rich in neutrophils and mediators that have a potential to delay neutrophil apoptosis [1], was used. Further evidence of altered survival of neutrophils in COPD airways was sought by studying the *ex vivo* anti-apoptotic effects of the sputum fluid phase on blood neutrophils.

METHODS

Subjects

A total of 20 subjects with COPD, 14 healthy smokers and 14 healthy control subjects were recruited (table 1). COPD was diagnosed according to Global Initiative for Chronic Obstructive Lung Disease (GOLD) guidelines [14]. All the COPD subjects and healthy smokers were current smokers, with mean 39 and 30 pack-yrs, respectively. Healthy smokers were free of chronic respiratory symptoms and had normal spirometry. Healthy control subjects were nonsmokers without significant past or current respiratory symptoms and with normal lung function. None of the subjects had experienced any signs of a respiratory infection in the 4 weeks preceding the study and none were treated with inhaled or oral steroids. None of the subjects had any other chronic disease.

The COPD subjects were classified into GOLD severity categories (stages 0–IV) on the basis of their post-bronchodilator forced expiratory volume in one second (FEV_1)/forced vital capacity (FVC) and FEV_1 % predicted. COPD stage 0 subjects (n=8) were current smokers with chronic cough and sputum production but normal lung function (FEV_1 >80% pred and FEV_1/FVC >0.7). The other COPD subjects were classified into GOLD stage I–IV severity categories if their post-bronchodilator FEV_1/FVC was <0.7 and their FEV_1 fell into set bands (stage 1: $FEV_1 \geqslant 80\%$; stage 2: $50\% \leqslant FEV_1 < 80\%$; stage 3: $30\% \leqslant FEV_1 < 50\%$; stage 4: $FEV_1 < 30\%$). Three subjects were in stage I, eight subjects were in stage II and one subject was in stage IV. Transfer factor of the lung for carbon monoxide ($D_{L,CO}$) was measured by standard methods [15].

Sputum samples for both cytological analyses and *in vitro* experiments with cultured neutrophils were obtained from all subjects. For *in vitro* experiments, neutrophils were isolated from four separate individuals with no smoking history or lung disease.

The study was approved by the ethics committees of the Southampton University Hospital Trust (Southampton, UK) and Helsinki University Hospital (Helsinki, Finland). All subjects gave full informed consent.

Sputum induction and processing

Sputum induction was conducted using the guidelines of the European Respiratory Society Task Force [16]. A standard procedure of induction was conducted using 4.5% hypertonic saline given at 5-min intervals for a maximum of 20 min, with mean induction times being similar in the three subject groups. The mucoid components of sputum were selected in order to reduce salivary contamination, and were processed as previously described [17] with slight modifications. Briefly, expectorated samples were divided into two parts: one part was treated by adding four volumes of PBS; the other was processed with four volumes of dithioerythritol (DTE). Both suspensions were filtered through 70-μm nylon gauze and centrifuged at $400 \times g$ at 4°C for 10 min. The DTE-processed samples were used to make cytospins for total and differential cell counts. The fluid phase of the PBS-processed samples was

TABLE 1 Patient characteristics

	COPD	Healthy smokers	Healthy controls
Patients n	20	14	14
Sex male/female	9/11	9/5	9/5
Age yrs	55 (41–72)	50 (22–64)	44 (20–62)
Smoking pack-yrs	39 (17–75)	30 (5–60)	
Post-bronchodilator FEV_1 % pred	78 (25–109)[#]	93 (72–110)	105 (86–128)
Post-bronchodilator FEV_1/FVC %	66 (28–87)**	78 (71–88)	79 (70–94)
$D_{L,CO}/V_A$ % pred	53 (28–82)**	75 (50–96)	Not done

Data are presented as n for patients and sex, as median (range) for age and smoking and as mean (range) for lung function. COPD: chronic obstructive pulmonary disease; FEV_1: forced expiratory volume in one second; % pred: % predicted; FVC: forced vital capacity; $D_{L,CO}$: transfer factor of the lung for carbon monoxide; V_A: alveolar volume. #: p<0.0001; **: p<0.01.

aliquoted and used for the *in vitro* apoptosis assays. The total protein contents of sputum supernatants were measured using the Bradford protein assay (Bio-Rad, Hercules, CA, USA) in order to adjust the *in vitro* results. For cell differential counts, 400 nonsquamous cells were counted on cytospins stained by the Diff–Quick method [18].

Assessment of apoptotic neutrophils

For *in vitro* assays only, neutrophil apoptosis was assessed by morphological criteria and flow cytometry. The morphological criteria were the disappearance of chromatin bridges between nuclear lobes (early apoptosis), and shrinkage or fragmentation of the nucleus (late apoptosis; fig. 1). The percentage of apoptotic neutrophils was assessed by counting 400 neutrophils. This was done by two independent observers with good interobserver reproducibility (intraclass correlation coefficient 0.80). The results were, therefore, expressed as the mean of two counts obtained by the two observers.

Apoptosis was also assessed by flow cytometry, using the technique of VERMES *et al.* [19], in which binding of Annexin V (AxV) detects phosphatidylserine externalised on the outer leaflet of the cell membrane of apoptotic cells. Briefly, neutrophils were washed twice in cold PBS and were then resuspended in binding buffer (10 mM HEPES pH 7.4; 140 mM NaCl; 2.5 mM $CaCl_2$) at a density of 1×10 cells·100 µL^{-1}, in 5 mL propylene fluorescence-activated cell sorting (FACS) tubes. AxV–phycoerythrin (PE; 1 µg·mL^{-1}) and 7-aminoactinomycin D (7-AAD; 2.5 µg·mL^{-1}) were added to the tubes, which were then incubated in the dark for 15 min. Following this, 300 µL cold binding buffer was added and cells analysed using a FACScan flow cytometer (Becton and Dickinson, Oxford, UK). Control tubes lacking AxV-PE, 7-AAD or both were included. Analysis of dot-plots of fluorescence channel (fl) 2 (AxV-PE) versus fl3 (7-AAD) was performed using WinMDI 2.8^{TM} (Scripps Research Institute, La Jolla, CA, USA). The degree of early apoptosis was shown as the number of AxV-positive/7-AAD-negative cells, expressed as a percentage of total cells. AxV-negative/7-AAD-negative cells were counted as viable cells, whereas AxV-positive/7-AAD-positive and AxV-negative/7-AAD-positive cells were counted together as late apoptotic and necrotic cells.

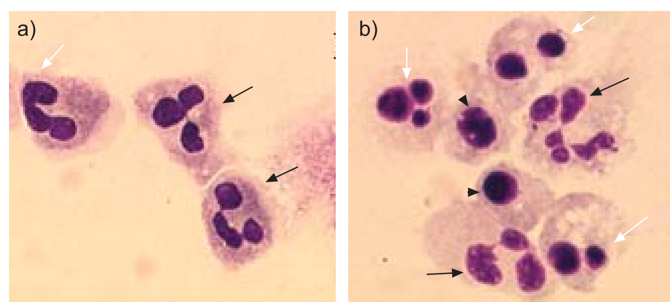

FIGURE 1. Sputum neutrophils. a) Normal neutrophils have at least two nuclear lobes interconnected by thin chromatin bridges (black arrows). Cells undergoing early apoptosis lose the chromatin bridges (white arrow). b) Neutrophils after 22 h in culture. Two normal neutrophils can be seen (black arrows). In cells undergoing late apoptosis (black arrowheads), there is considerable shrinkage of the cell nucleus due to condensation of nuclear chromatin. Occasionally, in late apoptotic neutrophils (white arrows), there is also fragmentation of the nucleus resulting in distinct apoptotic bodies.

Anti-apoptotic effects of induced sputum fluid phase on cultured blood neutrophils

The ability of sputum constituents to modulate neutrophil survival *in vitro* was investigated by culturing peripheral blood neutrophils purified from heparinised blood for 22 h (the time-point being identified in pilot experiments as being optimal) in the presence or absence of PBS-processed sputum fluid phase. A period of 22 h was chosen for all experiments because the signal is consistent at this time-point, with a median reduction in survival of 68% when neutrophil cells are incubated with sputum supernatants.

GM-CSF was used as a positive control because of its known ability to reduce apoptosis *in vitro* [8]. PBS was used as a negative control. All the sputum fluid-phase samples were pre-treated by irradiation with a dosage of 100 Gy to prevent bacterial growth which could affect the survival of neutrophils in culture. Heparinised venous blood was obtained from four healthy volunteers and neutrophils were isolated by dextran sedimentation/Lymphoprep (Axis-Shield, Oslo, Norway) centrifugation at $800 \times g$ for 25 min at 20°C. The removal of contaminating erythrocytes was achieved by hypotonic lysis. The remaining granulocytes were washed with culture medium at $400 \times g$ for 10 min at 4°C. The purity of the neutrophil population was routinely >96%. Cells were resuspended at 4×10^6 cells·mL^{-1} in RPMI 1640, supplemented with 20% volume/volume (v/v) foetal calf serum (FCS; TCS Biologicals, Buckingham, UK), 4 mM L-glutamine, 2 mM sodium pyruvate, 100 U·mL^{-1} penicillin and 100 µg·mL^{-1} streptomycin (all from Invitrogen, Paisley, UK). Neutrophils were then incubated in Falcon tubes (Becton and Dickinson) at 37°C in a 5% CO_2 atmosphere (final volume 0.5 mL; 4×10^6 cells). The experiments with cultured neutrophils were carried out in the presence or absence of: sputum supernatants of healthy controls, healthy smokers and patients with COPD; GM-CSF (10 ng·mL^{-1}; R&D Systems, Oxford, UK); and PBS as control. In preliminary studies, serial dilutions (1:10, 1:20 and 1:40) of sputum supernatants were tested for their effect on neutrophil survival in order to find the concentration which gives the maximum effect. The percentage of apoptotic cells in medium alone (in the absence of sputum supernatant) was 38.5%. This was reduced to 28–30% when using the 1:10 dilution, 21–30% when using 1:20 dilution and 33–35% when using the 1:40 dilution. The maximum reduction was, therefore, seen at the 1:20 dilution of sputum. Such effects were comparable to the anti-apoptotic actions of 10 ng·mL^{-1} lipopolysaccharide (which reduced the spontaneous apoptosis of neutrophils to 20.6%). This optimal dilution was, therefore, used in all subsequent experiments.

After 22-h culture, cells were harvested by centrifugation at $300 \times g$ for 10 min at 4°C, washed twice and resuspended in PBS containing 10% (v/v) heat-inactivated FCS. Apoptotic neutrophils were detected by morphology from cytospins and by flow cytometry as described above. The results were expressed as a percentage of the apoptosis observed in the PBS-treated samples, to account for variability between experiments.

TABLE 2 Induced sputum cell counts and apoptosis data from sputum cytospins

	COPD	Healthy smokers	Healthy controls	p-value
Patients n	20	14	14	
Total cell count $10^6 \cdot mg^{-1}$ sputum	2.8 (0.9–17)	2.2 (0.4–4.4)	1.8 (0.3–19)	NS
Viability %	84 (60–100)	91 (50–100)	92 (88–95)	NS
Neutrophils				
$10^6 \cdot g^{-1}$	0.54 (0.16–5.90)	0.51 (0.03–2.90)	0.41 (0.25–6.29)	NS
%	58 (36–89)	54 (28–79)	30 (0–61)	<0.0001
Macrophages				
$10^6 \cdot g^{-1}$	0.46 (0.08–1.27)	0.50 (0.07–2.26)	0.82 (0.23–5.6)	NS
%	37 (8.3–63)	43 (20–71)	63 (37–95)	0.001
Eosinophils %	0.2 (0–3.0)	0 (0–2.0)	0 (0–1.0)	NS
Lymphocytes %	0.5 (0–2.0)	0 (0–2.0)	0.4 (0–2.8)	NS
Apoptotic neutrophils				
$10^6 \cdot g^{-1}$	0.41 (0.04–1.8)	0.44 (0.02–1.11)	0.31 (0.05–3.93)	NS
%	36 (16–71)	36 (22–66)	34 (18–55)	NS

Data are presented as median (range), unless otherwise stated. Cell counts are expressed as absolute counts per weight of sputum and as percentages of total inflammatory cells, excluding squamous cells. COPD: chronic obstructive pulmonary disease; NS: not significant.

Statistics

As the data were not normally distributed, nonparametric tests were used. Data for individual variables from the several groups were first analysed using the Kruskal–Wallis test, followed by the Mann–Whitney U-test. Correlations between variables were sought using the Spearman rank correlation test. A p-value of <0.05 was considered statistically significant.

RESULTS

Sputum cell differential counts and sputum neutrophil apoptosis

Adequate sputum samples were obtained from all the subjects. The median percentages of squamous cell contamination were 2.5, 3.6 and 6.2%, respectively, for COPD patients, healthy smokers and healthy controls (p>0.5). Viabilities, as assessed by trypan blue, were 84, 91 and 92%, respectively, for COPD patients, healthy smokers and healthy controls (p>0.5). Subjects with COPD and healthy smokers had a significantly higher percentage than healthy control subjects of sputum neutrophils (p<0.0001 and p=0.01, respectively; table 2; fig. 2). COPD subjects also had a significantly lower percentage of sputum macrophages than healthy control subjects (p<0.0001). Compared with healthy smokers, subjects with COPD had a lower percentage of sputum macrophages (p=0.05) and a tendency towards a higher percentage of sputum neutrophils (p=0.06). However, there were no differences between the percentages of apoptotic neutrophils on sputum slides in the different subject groups (fig. 3). A sub-analysis was performed comparing subjects with established COPD with subjects with stage 0 COPD. No significant differences between these sub-groups were seen for any of the parameters of neutrophil apoptosis measured.

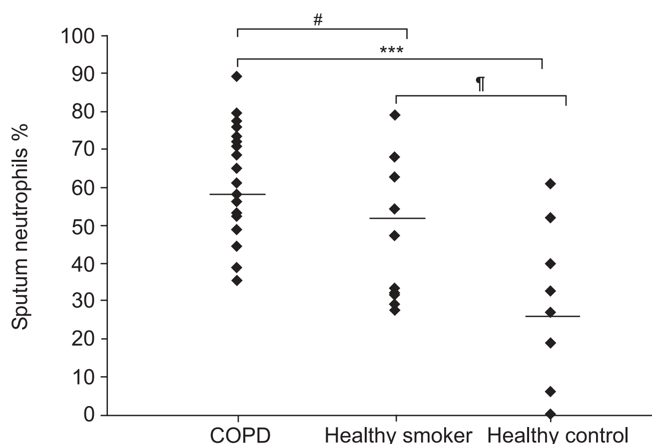

FIGURE 2. Sputum neutrophil counts (shown as percentages of nonsquamous cells). COPD: chronic obstructive pulmonary disease. #: p=0.06; ¶: p=0.01; ***: p<0.001.

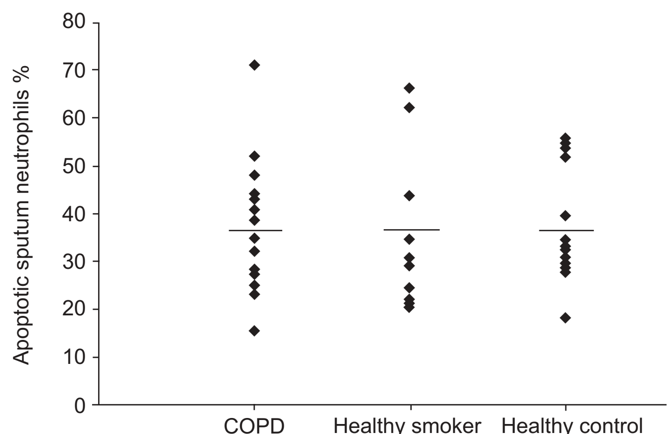

FIGURE 3. Sputum apoptotic neutrophil counts (shown as percentages of all neutrophils). Horizontal bars represent median values. COPD: chronic obstructive pulmonary disease.

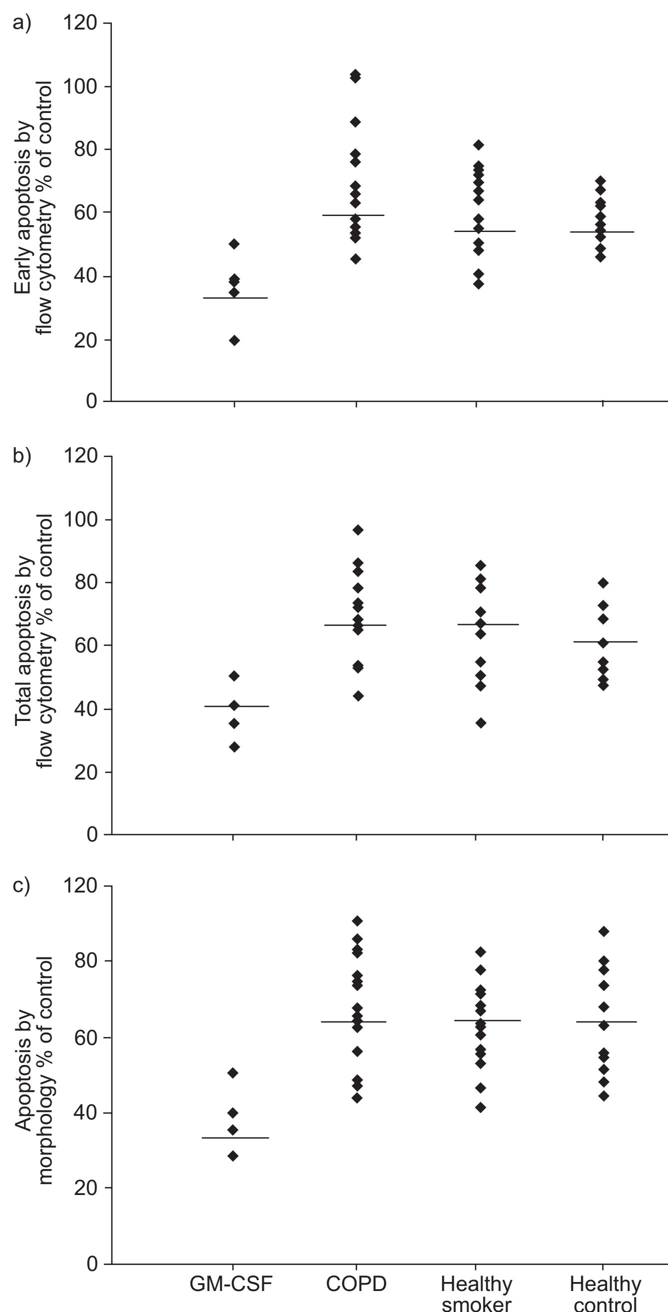

FIGURE 4. Neutrophil apoptosis after 22-h culture of blood-derived neutrophils with sputum supernatants from the three subject groups. a) Early apoptosis, measured by fluorescence-activated cell sorting (FACS); b total apoptosis, measured by FACS; and c) apoptosis, measured by morphology. Data are shown as percentages of apoptotic neutrophils seen in control culture medium and PBS alone. Horizontal bars represent median values. GM-CSF: granulocyte macrophage-colony stimulating factor; COPD: chronic obstructive pulmonary disease.

There was a significant negative correlation between pack-yrs smoked and FEV1 % pred (r_s=-0.47; p=0.005). Relative neutrophil counts correlated inversely with FEV1 % pred (r_s=-0.43; p=0.005) and *D*L,CO/alveolar volume (r_s=-0.45; p=0.05).

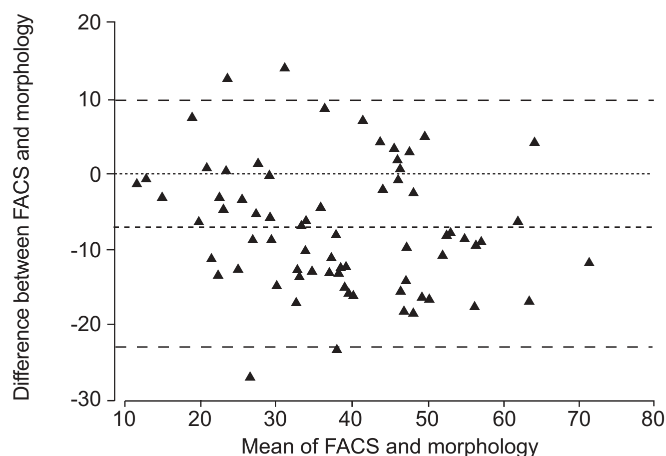

FIGURE 5. Bland–Altman plot for neutrophil apoptosis as assessed by fluorescence-activated cell sorting (FACS) and morphology. -----: mean difference between FACS and morphometry; – – –: upper and lower limits of agreement.

Anti-apoptotic effects of induced sputum extract on cultured blood neutrophils

All the sputum samples contained significant pro-survival activity for neutrophils, as demonstrated by the significant reduction in the numbers of apoptotic cells seen after 22 h of culture in the presence of the fluid phase of induced sputum when compared with spontaneous apoptosis in culture medium and PBS alone. However, there were no significant differences between the effects of the sputum fluid phase from nonsmoking controls, smokers and COPD patients on the extent of neutrophil apoptosis, whether assessed by low cytometry or morphology (fig. 4). There was a good correlation between the two methods (flow cytometry *versus* morphology) in detecting apoptosis (r_s=0.70; p<0.0001), and Bland–Altman analysis showed good agreement between the two methods (fig. 5). The positive control, GM-CSF, effectively reduced apoptosis as shown in figure 4.

The mean total protein content of sputum was not significantly different between the three groups (COPD: 259 $\mu g \cdot mL^{-1}$; healthy smokers: 253 $\mu g \cdot mL^{-1}$; healthy control subjects: 181 $\mu g \cdot mL^{-1}$), and when the apoptosis data were corrected for concentrations of total protein, there were still no significant differences in the anti-apoptotic activity of sputum between the subject groups.

DISCUSSION

This study suggests that airways neutrophilia in COPD is not a result of increased pro-survival activity in the airways, *i.e.* of reduced pro-apoptotic mechanisms, involving soluble inflammatory mediators present in the epithelial lining fluid. While this study shows that the sputum fluid phase, which samples the bronchial lining fluid of the larger airways, invariably contains significant activity that extends the survival of neutrophils *ex vivo*, this activity is no different in smokers who are healthy or who have COPD, or in healthy nonsmoking subjects.

A number of studies to date have sought to elucidate the mechanisms that promote the accumulation of granulocytes in

inflammatory airways diseases. Studies in asthma have shown that sputum contains fewer apoptotic eosinophils, the numbers of which correlate negatively with disease severity [20]. Further studies using the sputum fluid phase and either guinea pig [21] or human [22] eosinophils have attributed this effect to the cytokines GM-CSF and IL-5 [21] and prostaglandin E_2 [22]. Following the same rationale, the present authors have hypothesised that a similar mechanism could explain the airways neutrophilia that is typical of COPD. However, the present study found no significant differences in the percentages of apoptotic neutrophils in the sputum of subjects with COPD, healthy smokers and healthy control subjects. Furthermore, the rates of *in vitro* apoptosis of circulating neutrophils incubated with sputum supernatants obtained from all three groups were similar. Taken together, this strongly suggests that anti-apoptotic mechanisms, although operative within the airways, are not the basis of airways neutrophilia in COPD. Measuring factors such as IL-8 and GM-CSF was part of the original plan, but little justification was found to explore further which chemokines/cytokines are responsible for cell survival, since no differences in cell survival were found.

Eight COPD subjects with stage 0 COPD (*i.e.* chronic respiratory symptoms but normal lung function) and 12 subjects with more severe COPD (eight of whom had stage II COPD) were studied. There were no significant differences in the numbers of apoptotic neutrophils or the extent of *in vitro* apoptosis between stage 0 COPD and more severely afflicted patients. Moreover, the numbers of apoptotic neutrophils did not correlate with lung-function parameters. Thus, it seems that the anti-apoptotic activity in the airways is not a function of COPD severity, although this cannot be ruled out for the more severe stages III and IV COPD, since only one subject with stage IV was included in the current analysis.

Apoptosis is an essential mechanism for clearing neutrophils. During apoptosis, neutrophils retain their granule contents, lose the ability to secrete them in response to secretagogues, and lose their phagocytic capacity [8]. Together with the removal of apoptotic neutrophils by macrophages, this limits tissue injury during the resolution phase of inflammation. Inefficient clearance of apoptotic cells because of either less effective or absent macrophages results in secondary necrosis and release of neutrophil activation products that are potentially toxic to the lung [8]. While the present authors have found that sputum macrophage counts, expressed as a percentage of total inflammatory cell counts, were lower in patients with COPD than in healthy control subjects, this did not seem to affect clearance since the numbers of apoptotic neutrophils were no different in COPD. Phagocytosis of dying neutrophils by macrophages before they undergo secondary necrosis and release toxic metabolites limits tissue injury, inhibits the release of pro-inflammatory cytokines and results in the production of anti-inflammatory mediators [23]. It has been shown that neutrophil elastase can cleave the phosphatidylserine receptor and impair phagocytic recognition mechanisms [24]. It remains, therefore, to be elucidated whether in COPD more neutrophils undergo secondary necrosis as opposed to apoptosis, resulting in higher concentrations of neutrophil-derived mediators such as myeloperoxidase and neutrophil elastase [22, 23].

Previous studies have shown that *in vitro* neutrophil apoptosis of blood neutrophils from subjects with COPD occurs at a similar rate as in healthy individuals and smokers with normal lung function [12], but is reduced during acute exacerbations of COPD and returns to levels seen in healthy control subjects within 2 weeks [13]. These observations are in keeping with studies showing reduced apoptosis of blood and bronchoalveolar lavage neutrophils in acute lung inflammation, such as respiratory distress syndrome, pneumonia [25, 26] and exacerbations of bronchiectasis [27]. Future studies are needed to investigate the effects of COPD exacerbations on airway neutrophil survival.

The question of why sputum neutrophil counts are increased in COPD remains unanswered. The present authors have recently shown a significant correlation between raised neutrophil counts and reduced FEV1 across the spectrum of COPD severity [4], with both the airflow limitation and sputum neutrophilia being related to the degree of small airways dysfunction, as assessed by expiratory high-resolution computed tomography lung-density measurements. A recent study by HOGG *et al.* [3] showed prominent inflammation in the small airways in COPD, which correlated with the decline in lung function. It is, therefore, plausible that the sputum neutrophilia that is consistently reported reflects increased accumulation of neutrophils in the small airways and their transport by the mucociliary escalator into the larger airways that are sampled by sputum induction.

In conclusion, neutrophil survival is not significantly altered in the airways of subjects with stable chronic obstructive pulmonary disease, even though their sputum contains increased numbers of neutrophils. It remains to be seen whether neutrophil influx into the airways, directed by increased chemotactic activity, is responsible for this phenomenon and to what extent chronic obstructive pulmonary disease exacerbations might affect neutrophil survival.

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