Apoptosis of peripheral blood neutrophils in COPD exacerbation does not correlate with serum cytokines

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Summary The study investigated the relationship between apoptosis of peripheral blood neutrophils during exacerbation of chronic obstructive pulmonary disease (COPD) and the inflammatory response that characterises this condition. Twenty-six hospitalised patients with COPD exacerbation and 13 controls were included. Three sequential blood and sputum samples were obtained from patients at admission, after 3 days and at discharge. Blood apoptotic neutrophils were measured by flow-cytometry and light microscopy. Serum and sputum levels of IL-6, IL-8 and TNF-\(\alpha\) were determined by an immunoassay technique. We found a significantly reduced percentage of apoptotic neutrophils at the onset of COPD exacerbation which increased over time (1.1\(\pm\)0.4\% at admission vs. 2.4\(\pm\)0.4\% at discharge, \(P<0.0001\)). Patients presented no changes in serum cytokines neither during exacerbation nor in comparison to controls. In contrast, sputum levels of cytokines were significantly increased compared to serum levels. There was no significant correlation between the apoptotic neutrophils and the cytokine concentrations in serum or sputum. None of the clinical parameters, such as smoking, microbial infection, corticosteroids or hypoxemia showed a correlation with neutrophil apoptosis. No relationship could be found between the reduced percentage of apoptotic neutrophils in blood and serum concentration of IL-6, IL-8 and TNF-\(\alpha\) or other clinical parameters in patients with COPD exacerbation.

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Introduction

Exacerbations are typical events in patients with chronic obstructive pulmonary disease (COPD) and have been associated with an increased inflammatory response that contributes to continuous tissue damage and progressive bronchial obstruction.1-3 Neutrophils are key cells in the COPD-associated inflammatory processes. Patients with COPD exhibit enhanced activity in circulating neutrophils4 and a massive accumulation of neutrophils in the bronchial tree.5 The increased number of neutrophils found in sputum and bronchoalveolar lavage may be the result of an intense neutrophil migration from the blood vessels due to chemotactic factors and/or a consequence of a prolonged survival due to anti-apoptotic factors.6 The apoptotic process facilitates the removal of inflammatory cells and is essential for the termination of inflammation. An impaired apoptotic mechanism presumably contributes to the accumulation of the neutrophils and the development of a persistent inflammatory condition.7

Little evidence exists concerning the particulars and the regulation of the circulating neutrophil apoptosis in COPD, a pulmonary disease which has been shown by several investigations to have a clear systemic involvement.8 A recent study on neutrophil apoptosis in patients with community-acquired pneumonia showed that patients with underlying COPD present a significantly reduced proportion of apoptotic blood neutrophils in comparison to patients without comorbidities.9 Noguera et al.10 found that COPD patients in stable state have similar percentage of apoptosis in blood neutrophils in comparison to healthy controls but exhibit an increased expression of adhesion molecules on the surface of the apoptotic neutrophils indicating a previously higher level of activation. In a previous study, we investigated patients with COPD exacerbation and found a delayed apoptosis of peripheral blood neutrophils ex vivo at the onset of the exacerbation.11 Apoptosis recovered after 14 days, paralleling the clinical improvement and no differences were observed between COPD patients at discharge and the healthy controls, similar to the study of Noguera et al.

Based on these findings, we hypothesised that the distinctive pattern of apoptosis of the peripheral blood neutrophils in these patients is related to the increased inflammatory response that characterises COPD exacerbations. According to in vitro studies, some key cytokines involved in the local and systemic inflammation during exacerbation, such as IL-6, IL-8 and TNF-α, are also associated with the regulation of apoptosis.12-14

The present study aimed to investigate the relationship between apoptosis of peripheral blood neutrophils and the cytokines IL-6, IL-8 and TNF-α in patients with COPD exacerbation.

Material and methods

Patients

Twenty-six hospitalised patients with a documented diagnosis of COPD according to GOLD criteria3 and presenting an exacerbation were included. The exacerbation was defined by any combination of the following major criteria: worsening dyspnoea, increase in sputum volume, and increase in sputum purulence; additional minor criteria (upper respiratory tract infection in the last 5 days, fever, increased wheezing, increased cough) were also taken into account, as described elsewhere.15 Patients with new radiological pulmonary infiltrate, known HIV infection, immunosuppressive therapy (other than corticosteroids), evidence of bronchiectasis or tumour were excluded.

In all patients, treatment of COPD exacerbation consisted of inhaled bronchodilators, systemic corticosteroids (prednisolone 75 mg i.v. daily for 3 days, followed by oral therapy 1 mg/kg for 2 weeks), antibiotics (beta-lactams or fluoroquinolones), and oxygen if \(P_{O2}\) < 60 mmHg.

A group of healthy controls were also investigated. It has been suggested that aging is associated with changes in neutrophil apoptosis16; therefore we recruited only controls older than 60, in order to match the COPD group. Exclusion criteria for the control group were: fever or antibiotic prescription during the previous month, smoking, any chronic pulmonary, hepatic, cardiovascular, neurological, rheumatologic or renal condition, diabetes mellitus, immunosuppressive therapy (including current systemic corticosteroid treatment with >15 mg/day in the previous 3 weeks), known HIV infection, evidence of tumour at any site.

The study was approved by the local ethical committee and written informed consent was obtained from all patients and controls prior to enrolment.

Design

A prospective study was conducted over a period of 8 months. All patients were investigated at admission (before initiating any treatment), after 3 days and after 14 days. Blood and sputum samples were...
collected at each visit. The following parameters were recorded on admission: age, sex, weight, height, smoking habits, previous medication, signs and symptoms of respiratory infection, chest radiograph (to exclude pulmonary infiltrates), lung function tests, blood gases analysis and microbiological findings.

The controls were investigated once, at inclusion in the study, for neutrophil apoptosis and serum concentrations of cytokines.

**Measurement of apoptotic neutrophils**

Two methods have been used for measuring apoptosis: (1) flow cytometric measurement of Annexin V-PE/7AAD stained cells and (2) light microscopy to assess the nuclear morphology.

**Separation of neutrophils from peripheral blood**

The isolation of neutrophils from peripheral blood was performed as described previously. Briefly, neutrophils were separated by density gradient centrifugation at 470g for 25 min using Ficoll separating solution (Biochrom KG Berlin, Germany) followed by hypotonic lysis of erythrocytes. The cell suspension was washed and resuspended at a concentration of 1 x 10⁶ cells/ml in RPMI medium supplemented with 5.9 g HEPES, 2 g NaHCO₃, 50 ml FCS, 20 ml Penicillin-Streptomycin-Neomycin 2% solution, pH = 7.4. Only cell suspensions with > 96% neutrophils and a viability of > 95% as determined by trypan blue (Sigma, Taufkirchen, Germany) staining were considered for further analyses.

**Annexin V-PE/7AAD staining and flow cytometry**

This technique is based on the property of apoptotic cells to early redistribute the phosphatidylserine (PS) from the inner to the outer layer of cell membrane. As described elsewhere, during early stages of apoptosis a cell will stain with Annexin V, which has selective affinity for PS, but not with 7-amino-actinomycin D (7AAD), which stains the nucleus of cells with ruptured membranes.

Annexin V-PE/7AAD staining was performed with the Annexin V-PE Apoptosis Detection Kit I (PharMingen, Becton Dickinson, Heidelberg, Germany) according to manufacturer’s protocol. Briefly, the isolated neutrophils were washed and centrifuged in binding buffer (0.1M HEPES/NaOH (pH 7.4), 1.4M NaCl, 25mM CaCl₂) and the pellet was resuspended in 5μl Annexin V-PE, 5μl 7AAD and 100μl binding buffer, followed by incubation for 15 min at room temperature and in the dark. Measurement was performed within 15 min by a three-colour flow-cytometer (FACScan Becton-Dickinson, Heidelberg, Germany) using CellQuest software. A predetermined count of 10,000 granulocytes was set on forward light scatter. Annexin V-PE was read in FL-2 and 7AAD in FL-3. Only Annexin V-PE (+) cells with intact cell membrane, i.e. 7AAD (−), were considered as apoptotic and expressed as percentage of the total neutrophil count.

**Nucleus morphology and light microscopy**

Another assessable feature of apoptotic cells is the condensation and fragmentation of chromatin that can be observed by light microscopy. A cytopsin slide was prepared from the RPMI-suspended neutrophils (2 x 10⁶ centrifuged at 470g for 3 min) and stained with Pappenheim solution (Sigma, Taufkirchen, Germany). Two hundred cells were counted and morphologically evaluated by light microscopy. Apoptotic neutrophils were identified by a condensed single-lobed or a completely fragmented nucleus, and were expressed as percentage of the total neutrophil count.

**Measurement of IL-6, IL-8, TNF-α in serum and sputum**

Blood samples were centrifuged at 1400g for 10 min; the serum was aliquoted and stored at −70°C until measurement.

Spontaneous sputum samples were collected from COPD patients in a sterile pot after rinsing the mouth with water. A sputum smear for Gram’s stain was prepared and neutrophils and squamous cells were counted under low-power microscopy (× 100) and samples with > 25 neutrophils and < 10 squamous cells per field were considered valid for further microbial and cytokine investigation. The weight of the sample was recorded. The sputum was then mixed with equal volume of dithiothreitol (Sputolysin¹⁰ Reagent, Calbiochem, Merck Biosciences GmbH, Schwalbach, Germany), diluted 1:10 in NaCl 0.9% and placed in a shaking water bath at 37°C for 15 min, during which the sample was further homogenised every 5 min. After filtering through gauze to remove mucus and debris and centrifugation at 470g for 10 min, the supernatant was aliquoted and stored at −70°C until measurements.

IL-6, IL-8 and TNF-α in serum and sputum were measured by a solid phase Enzyme Amplified Sensitivity Immunoassay (BioSource Europe SA, IBL Hamburg, Germany) and expressed in pg/ml. The minimum detectable concentrations were 2 pg/ml for IL-6, 0.7 pg/ml for IL-8 and 3 pg/ml for TNF-α.
Sputum supernatant was diluted at 1:50 for detection of IL-8 and at 1:4 for detection of TNF-α.

**Statistical analysis**

Data processing and analysis were performed using SPSS 10.0 for Windows (SPSS, Chicago, IL, USA). Normally distributed data are expressed as percentage and mean ± SD, categorical data as percentage and non-normally distributed data as median and interquartile range. The parametric distribution was confirmed by the Shapiro-Wilk test. Apoptotic neutrophils at each measurement were contrasted using paired t-test. Cytokines levels at each measurement were compared using Wilcoxon test. The correlation between the two methods of measurement and the correlation between apoptotic neutrophils and cytokines level (all time-points) were evaluated by Spearman’s coefficient. The comparison of apoptosis percentages and cytokine levels between patient groups, categorised according to clinical parameters, was performed by the unpaired t-test and Anova test with post-hoc Bonferroni correction. A P value of <0.05 was considered as significant.

**Results**

**Baseline characteristics**

Twenty-six patients with COPD exacerbation were included in the study. The baseline characteristics are shown in Table 1. The control group consisted of 13 subjects (mean age 67.9 ± 3.3 years; seven men and six women).

**Levels of apoptotic neutrophils in peripheral blood**

In contrast to changes in core morphology due to chromatin condensation and fragmentation, the externalisation of phosphatidylserine (measured by using the binding protein Annexin V) is an early feature of apoptosis. Therefore, the apoptotic rates measured by Annexin V-PE/7AAD staining were discretely higher than those observed by core morphology evaluation (data not shown). However, the two methods used for the measurement of neutrophil apoptosis showed a good correlation (ρ = 0.747, P = 0.001, all time-points) and the values provided by flow-cytometry have been further considered in the statistical analysis.

Figure 1 shows the mean percentage of apoptotic neutrophils in patients with COPD exacerbation and in controls. There was a statistically significant difference between the percentage of apoptotic neutrophils at admission vs. day 3 (1.1 ±0.4% vs. 1.9 ± 0.8%, P = 0.01) as well as at admission vs. day 14 (1.1 ± 0.4% vs. 2.4 ± 0.8%, P < 0.0001) in COPD patients. Furthermore, COPD patients presented a significantly lower apoptosis percentage at admission compared to controls (1.1 ± 0.4% vs. 2.1 ± 0.3%, P < 0.0001), while the values of day 14 were similar to those of controls (P = 0.1).

**Serum and sputum levels of cytokines**

Figure 2 shows the levels of IL-6, IL-8 and TNF-α in serum of controls and COPD patients. No increase in
between admission and day 14 was possible. An accurate statistical comparison of sputum levels after treatment in more than 70% of patients, no significant correlation could be observed between apoptotic neutrophils and serum IL-8 and TNF-α (Table 3).

We also tested for a possible relationship between the percentage of apoptotic neutrophils and the sputum concentration of the measured cytokines but no significant correlation could be observed.

Correlation of neutrophils apoptosis with clinical parameters

Patients were categorised according to recorded clinical parameters which could possibly have an impact on neutrophil apoptosis. No differences in the level of apoptotic neutrophils at admission were found in respect to the severity of bronchial obstruction (FEV1 < 35% 1.1 ± 0.5% vs. FEV1 ≥ 35%, P = 0.6), BMI (inactivity vs. BMI ≥ 25), smoking habits (current smokers 1.4 ± 0.6% vs. ex-smokers 1.0 ± 0.4%, P = 0.1), corticosteroid treatment prior to admission (oral 1.1 ± 0.7% vs. inhaled 1.2 ± 0.2%, P = 0.9; oral 1.1 ± 0.7% vs. steroid naïve 0.9 ± 0.3%, P = 0.3) or hypoxemia (PO2 < 60 mmHg 0.8 ± 0.3% vs. PO2 ≥ 60 mmHg 1.2 ± 0.3%, P = 0.06).

Discussion

Our study revealed a reduced percentage of apoptotic blood neutrophils in patients with COPD exacerbation. This reduction in apoptotic neutrophils was not related to serum and sputum concentrations of IL-6, IL-8 and TNF-α or to other specific clinical parameters.

In a previous investigation we showed that blood neutrophils of patients with COPD exacerbation exhibited a delayed spontaneous apoptosis ex vivo which was progressively restored after treatment and clinical remission.11 This finding has been confirmed by the present study as well as by another recent investigation9 and may be a feature of the non-specific immune response against the triggers of exacerbation. Blood neutrophils have a short life span in the circulation (6–12 h) and are usually removed through genetically programmed apoptosis. However, the normal survival may be due to their serum levels: IL-6 837 pg/ml; IL-8 11.3 pg/ml; TNF-α 11.1 pg/ml. Furthermore, there was a significant change in serum level of cytokines during the course of exacerbation. When stratifying the COPD patients according to corticosteroid treatment prior to hospitalisation, no differences were observed between the groups with oral, inhaled or naïve steroids with respect to serum IL-6 (P = 0.8), IL-8 (P = 0.3) and TNF-α (P = 0.8).

In contrast, increased levels of cytokines were observed in sputum of COPD patients at the onset of the COPD exacerbation (Table 2) in comparison to their serum levels: IL-6 837 pg/ml vs. 13.4 pg/ml, P = 0.001; IL-8 11.7 pg/ml vs. 3.6 pg/ml, P = 0.001; TNF-α 134 pg/ml vs. 11.1 pg/ml, P = 0.002. Because the expectoration improved after treatment in more than 70% of patients, no accurate statistical comparison of sputum levels between admission and day 14 was possible.

Correlation of apoptotic neutrophils with sputum and serum cytokines

We tried to correlate the percentage of apoptotic neutrophils to the level of serum cytokines, assuming that a small, undetectable increase of these proinflammatory cytokines may result in a marked reduction of apoptosis at the onset of exacerbation. Only IL-6 presented a discrete negative correlation with apoptotic neutrophils (r = −0.237, P = 0.032) while no correlation could be observed between apoptotic neutrophils and serum IL-8 and TNF-α (Table 3).
prolonged up to 48 h under certain conditions such as elevated blood concentration of cytokines, hormones or glucocorticoids.

Dunican et al.\textsuperscript{13} showed that TNF-\(\alpha\) stimulates neutrophil apoptosis during the first hours, while prolonged exposure has an inhibitory effect mediated by the anti-apoptotic chemokine IL-8. Biffl et al.\textsuperscript{14} reported that IL-6 also delays neutrophil apoptosis in vitro. A study focusing on the neutrophils apoptosis in patients undergoing coronary bypass found that apoptosis was suppressed postoperatively but the apoptotic percentage increased.

![Figure 2](image_url) \textbf{Figure 2} The levels of IL-6, IL-8 and TNF-\(\alpha\) in serum in patients with COPD exacerbation and in controls. The horizontal line is the median value; the box is the interquartile range, excluding outlying and extreme values. Neither between the three sequential measurements nor in comparison to controls statistically significant differences could be detected for any of measured cytokines.

\begin{table}[h]
\begin{tabular}{|c|c|c|c|}
\hline
Cytokine & Admission & Day 3 & Day 14 \\
& \((n = 26)\) & \((n = 15)\) & \((n = 7)\) \\
\hline
IL-6 & 837 (193–2266) & 643 (525–2136) & 47.0 (36.0–385) \\
IL-8 & 11,755 (7370–23,900) & 10,870 (8305–17,680) & 2850 (836–8317) \\
TNF-\(\alpha\) & 134 (22.8–538) & 14.0 (12.0–14.8) & 12.0 (12.0–14.0) \\
\hline
\end{tabular}
\caption{The levels of IL-6, IL-8 and TNF-\(\alpha\) in sputum in patients with COPD exacerbation.}
\end{table}

Data given as median value and interquartile range, expressed in pg/ml.

IL-6: interleukin-6; IL-8: interleukin-8; TNF-\(\alpha\): tumor necrosis factor-\(\alpha\).
Furthermore, in a study of Wedzicha et al.\textsuperscript{20} on cytokines,\textsuperscript{17}ing an important anti-apoptotic role for these after plasma depletion of IL-6 and IL-8, suggest-

An increased systemic inflammatory reaction in COPD patients has been proposed as an explanation for systemic hypoxemia\textsuperscript{18} and weight loss.\textsuperscript{19} Furthermore, in a study of Wedzicha et al.\textsuperscript{20} on 67 COPD patients, serum IL-6 was found to be slightly but significantly increased during exacerbation compared to stable state. These results could not be reproduced by our study. One possible explanation for this lack of increase in serum cytokine concentrations could be that we only enrolled 26 patients. This number of patients maintained enough statistical power to detect differences in the proportion of apoptotic neutrophils and sputum cytokine concentrations, but it might have not been sufficient enough to detect the much more moderate differences in serum cytokine levels, if they should exist. However, our study shows clearly that the pronounced decrease in apoptotic neutrophils at the onset of a COPD exacerbation cannot be related to IL-6, IL-8 and TNF-\textgreek{z} serum concentration. In fact, an interesting study of Murray et al.\textsuperscript{21} shows that LTB\textsubscript{4}, a proinflammatory molecule that can be synthesised and released by neutrophils, induces in vitro a powerful activation of granulocytes but, contrary to the current opinion, has little impact on the cell survival. The authors are suggesting that the granulocyte activation and the respective granulocyte-released inflammatory agonists do not appear to be critical for the delay of granulocyte apoptosis and that these events are not necessary directly related.

A limitation of our study is that no measurements of GM-CSF and G-CSF were performed; these mediators have been associated with an increase in neutrophils in peripheral blood, nasal and respiratory secretions during viral exacerbation of asthma\textsuperscript{22} and they are also involved in the regulation of neutrophil apoptosis.\textsuperscript{23}

Several studies are reporting important changes in the inflammatory markers in sputum in COPD patients during acute exacerbations.\textsuperscript{24,25} We also found high levels in sputum cytokines at the onset of the exacerbation, when compared to serum level, which decreased during the course of exacerbation, paralleling the clinical resolution. However, no correlation could be observed between the behaviour of measured sputum cytokines and that of the apoptotic neutrophils in peripheral blood, suggesting again that the two events are probably triggered and controlled by different factors. On the other hand, the measurements of cytokines in sputum exhibit variability due to several factors such as the temperature of processing, the nature of sputum, the effect of dithiothreitol etc.\textsuperscript{26} Although during our study the processing of sputum was uniform, technical limitations cannot be excluded and should be considered as a possible reason for these discrepancies.

Some other factors may be responsible for the reduced apoptosis of neutrophils in COPD exacerbation. Aoshiba et al.\textsuperscript{27} reported a strong anti-
apoptotic effect of nicotine on neutrophils in vitro; however, we found no significant difference between the percentage of apoptotic neutrophils in smokers vs. ex-smokers. This may be explained by the fact that the current smokers in our study discontinued or reduced smoking within the previous days because of the exacerbation of their symptoms, so that nicotine was practically re-moved from their blood and had no immediate influence on neutrophil apoptosis by the time of first measurement. Similarly, a recent study by Hodge et al.\textsuperscript{28} found no differences in apoptosis of airway cells and BAL-derived T-cells between smokers and non-smokers COPD patients. However, it has been shown that an impaired neutrophils gene expression in smokers is maintained for at least 8 weeks after smoking cessation,\textsuperscript{29} suggesting that the effects of smoking on neutrophils are far more persistent and complex in vivo compared to in vitro.

Some bacterial products, such as lipopolysaccharide, have been also shown to prolong the survival of neutrophils in vitro studies\textsuperscript{30} and in animal model,\textsuperscript{31} through complex mechanisms, including activation of NF-kappa B and expression of anti-apoptotic proteins. Taking into account this evidence, we also analysed the relationship

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Serum</th>
<th>P value</th>
<th>Sputum</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-6</td>
<td>r</td>
<td>0.237</td>
<td>0.032</td>
<td>-0.185</td>
</tr>
<tr>
<td>IL-8</td>
<td>r</td>
<td>0.135</td>
<td>0.225</td>
<td>-0.127</td>
</tr>
<tr>
<td>TNF-\textgreek{z}</td>
<td>r</td>
<td>-0.127</td>
<td>0.074</td>
<td>-0.146</td>
</tr>
</tbody>
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IL-6: interleukin-6; IL-8: interleukin-8; TNF-\textgreek{z}: tumor necrosis factor-\textgreek{z}; r: Spearman’s bivariate correlation coefficient.
between the microbial etiology of COPD exacerbation and the neutrophil apoptosis. No significant difference in the percentage of apoptotic neutrophils was found in our population with regard to the presence or absence of microbial growth in sputum, probably because the infection was confined to the airways without massive endotoxemia or bacteremia.

There is overwhelming in vitro evidence showing that glucocorticoids attenuate neutrophil apoptosis and a recent in vivo study in bovine32 reported that dexamethasone administered to steers resulted in a statistically significant decrease in Fas mRNA abundance in blood neutrophils with subsequent prolonged cell survival. In contrast to this finding, the percentage of apoptotic neutrophil in COPD patients in our study constantly increased over time although all these patients received intravenous followed by oral steroids. Furthermore, there was no difference in percentage of apoptotic neutrophils at admission between patients on previous treatment with oral versus inhaled steroids or steroid naive. In a similar context, a study on apoptosis of airway epithelial and T-cells in COPD patients found that inhaled glucocorticoids did not significantly influence the survival of these cells, which was partly explained by the heterogeneity of the type of drug, dose and compliance to therapy.28

Hyoxia is another factor that may induce the neutrophil survival through the hypoxia-inducible factor (HIP1-α) present on the neutrophils.33 The study of Tamura et al.34 showed that acute hypoxemia enhances the inflammatory response and delays the apoptosis of circulating neutrophils. However, no differences in the apoptotic percentage of neutrophils were observed in our patients with respect to PO2 levels, maybe because such a dramatic hypoxemia was not noted in any of them. In conclusion, we believe that our investigation showing the impairment of systemic neutrophils apoptosis in COPD exacerbation provides a novel finding that supports the concept of COPD as a systemic disease. The surprising lack of relationship of this phenomenon with important disease-related factors, such as cytokines, smoking, obstruction, hyoxemia or glucocorticoids, raises a number of questions which deserve future research to clarify the significance and regulation of this process.

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