Neutrophil survival-enhancing activity in sputum from patients with diffuse panbronchiolitis

Katsushi Nishimaki\textsuperscript{a}, Jun Nawata\textsuperscript{b}, Shinji Okada\textsuperscript{a}, Toshio Hattori\textsuperscript{a}, Isao Ohno\textsuperscript{c,*}

\textsuperscript{a}Department of Respiratory and Infectious Diseases, Tohoku University Graduate School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan
\textsuperscript{b}Cardiovascular Medicine, Tohoku University Graduate School of Medicine, 1-1 Seiryo-machi, Aoba-ku, Sendai 980-8574, Japan
\textsuperscript{c}Department of Pathophysiology, Tohoku Pharmaceutical University, 4-4-1 Komatsushima, Aoba-ku, Sendai 981-8558, Japan

\textbf{Summary} Diffuse panbronchiolitis (DPB) is a life-threatening airway disease in which neutrophils persistently and massively emigrating to the airways cause progressive and irreversible tissue damage. However, the pathogenesis of the airway inflammation remains unclear. The failure of non-inflammatory removal of emigrating neutrophils due to delayed apoptosis has been proposed as a mechanism by which the neutrophilic inflammation persists. Therefore, we aimed to investigate whether an activity that delays neutrophil apoptosis is present at the inflamed sites in DPB. Neutrophils isolated from normal volunteers were cultured with sputum extracts of patients with DPB, and viability and apoptosis of neutrophil was evaluated for the culture period. Neutrophils cultured with sputum extracts for 2 and 3 days showed significantly enhanced survival compared to those with medium alone. The neutrophil survival-enhancing activity in sputum extracts was heat-labile and partially, but significantly, neutralized with anti-human GM-CSF, but not with anti-human G-CSF antibody. The enhancement of neutrophil survival was associated with an inhibition of apoptosis demonstrated by cytology, TUNEL assay and DNA fragmentation analysis. These results suggest that neutrophil apoptosis is prevented by survival-enhancing factors including GM-CSF in the airways of DPB, leading to neutrophil death by necrosis that causes further recruitment and activation of neutrophils.

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\textbf{KEYWORDS}
Neutrophils; Sputum; Diffuse panbronchiolitis; Survival; Apoptosis

Corresponding author. Tel.: +81 22 234 4183x5413; fax: +81 22 275 2013.
E-mail address: 1ohn@tohoku-pharm.ac.jp (I. Ohno).

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Introduction

Diffuse panbronchiolitis (DPB) was first described by Homma and coworkers as a chronic inflammatory disorder of the airways histologically characterized by peribronchiolitis with the infiltration of lymphocytes and plasma cells.\(^1\)\(^,\)\(^2\) DBP is not uncommon in Japan and largely restricted geographically to the Far East. A small number of patients have been confirmed in Korea\(^3\)\(^,\)\(^4\) and China.\(^5\) Only sporadic cases have been reported in US 6–11 and Europe.\(^9\)\(^,\)\(^10\) Some genetic component is suggested by a frequent HLA haplotype, HLA B54, in patients with DPB.\(^1\)\(^,\)\(^2\) Cystic fibrosis (CF) is the most common recessively inherited disorder in Caucasian populations that is caused by the mutation of a single gene coding CF transmembrane conductance regulator (CFTR). CFTR dysfunction predisposes to a wide range of multi-organ clinical manifestations including the respiratory tract.\(^12\)\(^,\)\(^13\) Although the predisposing factors to DPB and CF are different, the two diseases have similar features in terms of chronic neutrophilic airway inflammation and a high rate of respiratory infection with mucoid Pseudomonas aeruginosa (P. aeruginosa). A persistent and abundant influx of neutrophils to the airway lumen cause progressive and irreversible damage in airway tissues by releasing histotoxic mediators such as reactive oxygen species and proteolytic enzymes. Respiratory failure is the prime cause of morbidity and mortality in both disorders.\(^2\)\(^,\)\(^12\)\(^,\)\(^13\) Although the overexpression of cytokines and chemokines, such as TNF, IL-1 and IL-8, and the infection with P. aeruginosa in the airways are thought to be involved in the induction of neutrophilic airway inflammation,\(^2\)\(^,\)\(^14\) the mechanisms underlying the chronic bronchial inflammation are not fully understood.

Neutrophils have a very short life in the circulation (8–20 h) and in tissues in the absence of inflammation (1–2 days). Aged neutrophils undergo apoptosis (programmed cell death) prior to the phagocytic removal by macrophages. This removal does not cause the release of toxic mediators from the neutrophils. Once neutrophils influx to targeted sites as effector cells they are activated and local inflammatory mediators delay their apoptotic death. Activated neutrophils die by necrosis and release their toxic mediators. For the resolution of inflammation, apoptosis followed by safe removal by phagocytes of neutrophils is important. Conversely, the failure of non-inflammatory clearance of emigrating neutrophils due to the prevention of their apoptosis would result in persistent inflammation and excessive tissue damage as in DBP and CF. Many factors such as granulocyte-macrophage colony-stimulating factor (GM-CSF), granulocyte colony-stimulating factor (G-CSF) and lipopolysaccharide (LPS), found abundantly at inflammatory sites, have been demonstrated to be able to suppress neutrophil apoptosis.\(^15\)\(^–\)\(^18\)

Therefore, we hypothesized that the inhibition of apoptosis by factors at the site of inflammation render emigrating neutrophils necrotic, increasing the airway inflammation and causing it to persist in DBP. To access our hypothesis, we investigated whether an activity that enhances the survival of neutrophils is present at the inflamed sites by examining the effect of extracts of sputum from patients with DPB on the survival of neutrophils isolated from peripheral blood.

Materials and methods

Patients studied

We collected sputum expectorated spontaneously from 8 outpatients with DPB (Table 1). DBP was defined based on the clinical diagnostic criteria established by Homma and Yamanaka in 1969 in collaboration with the Ministry of Health and Welfare of Japan.\(^19\) All patients were non-smokers and had been treated continuously with theophylline, \(\beta_2\)-agonists and expectorants. Their symptoms were chronic cough and sputum with occasional exacerbations. During the month preceding the study, none of the patients had had an acute exacerbation, nor had been treated with antibiotics or corticosteroids. Macrolides were not given to the patients during the study period. By sputum culture, the mucoid type of P. aeruginosa was invariably detected.

Processing of sputum from patients

Sputum was collected in sterile tubes, kept cold and, immediately after the expectoration, processed for the culture of P. aeruginosa, the analysis of sputum cells and the collection of sputum extracts. Sputum culture was performed as previously described.\(^20\) P. aeruginosa was identified with an Enteric/Nonfermenter ID Kit (Becton Dickinson and Company, Sparks, Maryland) according to the manufacturer’s protocol. After the homogenization with sputasol (Unipath Ltd., Basingstoke, UK), cell differentials were counted and the viability of sputum cells was assessed by exclusion of trypan blue dye as previously described.\(^21\) Sputum extracts were collected as previously described\(^22\) with some modifications.
Briefly, the purulent portion of sputum was transferred to a tube followed by the addition of one volume of physiological saline. After stirred with a vortex mixer for 2 min, the sputum was ultracentrifuged at 4°C for 90 min at 40,000 g. Then, the supernatant was collected, passed through a 0.2-μm filter (Minisart) (Sartorius AG, Göttingen, Germany), and stored as the sputum extracts at −20°C.

**Neutrophil isolation and culture conditions**

Heparinized venous blood was obtained from healthy volunteers. Neutrophils were isolated by dextran sedimentation and centrifugation on a Ficoll-Paque (Amersham Pharmacia Biotech, Uppsala, Sweden) as previously described23 with some modifications. Neutrophils (>96% pure, >98% viable, by exclusion of trypan blue dye) were resuspended at 10^6 cells/ml in RPMI 1640 (GIBCO BRL, Life Technologies, Grand Island, NY) supplemented with 10% fetal bovine serum (Cansers International Inc., Toronto, Canada), and incubated in 24-well culture plates (Becton Dickinson Labware, Franklin Lakes, NJ) (1 ml/well) for three days at 37°C in a humidified atmosphere of 5% CO₂ in air. The cultures were performed in triplicate in the presence or absence of sputum extracts at the indicated concentration, recombinant human GM-CSF (TECHNO Corporation, Minneapolis, MN) (500 μg/ml) or LPS (from *Escherichia coli* 026:B6, Sigma Chemical Co., St. Louis, MO) (1 μg/ml) as a positive control. In some experiments, sputum extracts and LPS were preincubated at 4 or 85°C for 15 min, or sputum extracts and human GM-CSF were preincubated overnight at 4°C with an anti-human GM-CSF (TECHNO Corporation) (10 μg/ml) or an anti-human G-CSF (10 μg/ml) (R&D System, Inc., Minneapolis, MN) mouse monoclonal IgG1 antibody. The concentrations of antibodies used here were sufficient to neutralize 500 μg/ml of GM-CSF or 125 ng/ml of G-CSF, respectively, based on the information provided by the manufacturer. These concentrations of GM-CSF and G-CSF have been reported to inhibit neutrophil apoptosis by 50–70%.23,24 At each time point, after gentle resuspension, viable cells were counted by exclusion of trypan blue dye for each well in a blind fashion. Viability was calculated by the following formula: viability (%) = [the number of viable cells /that of viable cells at the beginning of culture (10^6 cells)] × 100. The mean value of triplicate determinations was used as that for each condition. For each condition examined, cell smears were prepared from triplicate incubations with cytospin (Cytospin 2, Shandon, Runcorn, UK) for further examination.

**Evaluation of neutrophil apoptosis**

Neutrophil apoptosis was evaluated by cytology, terminal transferase dUTP nick end-labeling (TUNEL) assay, and DNA fragmentation analysis. Cytospin preparations of neutrophils were stained with a Diff-Quick stain set (Baxter, Miami, FL), and examined under light microscopy at a magnification of 1000 in a blind fashion. Apoptotic neutrophils were defined as cells containing one or more characteristic darkly stained pyknotic nuclei.25 Results were expressed as percentage of apoptotic cells in more than 500 cells counted on duplicate slides for each condition. TUNEL assay was performed using an ApopTag Apoptosis Detection kit.
(Serologicals Corporation, Norcross, GA) according to the manufacturer’s protocol. Using an Automatic Nucleic Acid Isolation System (NA-1000) (KURABO, Osaka, Japan), DNA was extracted from neutrophils cultured for 24 h with medium alone, sputum extracts or LPS as mentioned above. Two micrograms of each DNA sample and a molecular size marker (1kb DNA Ladder, GIBCO BRL) were heated at 60°C for 10 min and then electrophoresed through 1.8% agarose (Nippon Gene Co. Ltd., Toyama, Japan) gel. After staining in ethidium bromide (Sigma) (10 μg/ml) the gel was viewed under UV light.

**Statistical analysis**

Values are presented as mean ± SEM. Mann–Whitney U-test was used to compare the differences between the values except for the values in the time-course experiments in which the differences were estimated with one-way analysis of variance and Fisher’s PLSD method. These analyses were performed using StatView 4.11 (Abacus Concepts, Berkeley, CA) for Macintosh. The level of significance was taken at P < 0.05.

**Results**

**Enhancement of neutrophil survival by sputum extracts**

The viability of neutrophils cultured with medium alone was 72.7 ± 9.5% at day 1, 36.7 ± 5.7% at day 2 and 10.2 ± 3.3% at day 3 (n = 6). In contrast, although not significantly different at day 1 (95.0 ± 6.9%, n = 8), the viability of neutrophils cultured with sputum extracts (10%) was significantly higher than that with medium alone at days 2 (74.1 ± 6.9%, n = 8; P < 0.01) and 3 (43.9 ± 7.2%, n = 8; P < 0.01), and similar to that with LPS (94.7 ± 5.5% at day 1, 86.9 ± 14.4% at day 2 and 45.4 ± 9.9% at day 3, n = 6) (Fig. 1). The effect of the sputum extracts on neutrophil survival was also dose dependent (Fig. 2). The viability of neutrophils at day 2 was significantly enhanced in the presence of concentrations of sputum extracts of 5 (54.5 ± 8.5%, n = 8; P < 0.05), 10 (76.4 ± 7.2%, n = 8; P < 0.01) and 20% (89.0 ± 11.5%, n = 8; P < 0.01) compared with that in medium alone (38.1 ± 6.0%, n = 6), but not significantly different in the presence of 2.5 (48.3 ± 6.9%, n = 8) and 1.25% (34.2 ± 9.1%, n = 8).

**Characterization of the neutrophil survival-enhancing activity in sputum extracts**

To examine the heat-stability of the neutrophil survival-enhancing activity in the sputum extracts, the neutrophils were cultured for 2 days with sputum extracts at various concentrations (n = 6 at 0% and n = 8 at the other concentrations). Viability was evaluated as described in 'Materials and methods’, and expressed with SEM. *P < 0.05 and P < 0.01 compared to 0%.
(75.5 ± 9.5%, n = 6) did not show any effect at all on the neutrophil survival-enhancing activity by LPS (70.0 ± 5.6%, n = 6), as expected. The neutrophil survival-enhancing activity in the sputum extracts was also inhibited partially but significantly by the preincubation with anti-GM-CSF antibody (52.2 ± 3.3%, n = 8, P < 0.05), but not with anti-G-CSF antibody (68.5 ± 3.5%, n = 8) (Fig. 3). Anti-GM-CSF antibody did not show any inhibitory effect on the viability of cells cultured with medium alone (36.3 ± 3.9%, n = 4). The increased viability of neutrophils by GM-CSF (71.8 ± 3.0%, n = 4) was completely abolished by anti-GM-CSF antibody (33.3 ± 2.7%, n = 4, P < 0.01).

Inhibition of neutrophil apoptosis by sputum extracts

To examine whether prolongation of neutrophil survival was due to the inhibition of apoptosis, cytology, TUNEL staining and DNA fragmentation analysis were performed. Based on the cell morphology, 53.3 ± 6.6% (n = 6) of cells were apoptotic 24 h after the culture with medium alone (Fig. 4A). In contrast, the percentage of apoptotic cells in neutrophils cultured with 10% sputum extracts was significantly reduced (11.0 ± 2.7%, n = 8, P < 0.01) (Fig. 4B), which was similar to that with LPS (11.0 ± 3.5%, n = 6). The number of cells positive for TUNEL staining was apparently decreased in the culture with sputum extracts (Fig. 4D) compared with those without sputum extracts (Fig. 4C). DNA fragmentation with the characteristic ‘ladder’ of DNA fragments in multiples of 180 bp, which was clear in neutrophils cultured with medium alone, was not as apparent in those with sputum extracts such as with LPS (Fig. 5).

**Figure 3** Characterization of neutrophil survival-enhancing activity in sputum extracts. Neutrophils (10⁶/ml) were cultured for 2 days with medium alone (n = 6), LPS (1 μg/ml) (n = 6) and sputum extracts (10%) (n = 8) with or without heating, and sputum extracts (10%) (n = 8) preincubated with anti-GM-CSF (10 μg/ml) or anti-G-CSF (10 μg/ml) antibody. Viability was evaluated as described in ‘Materials and methods’, and expressed with SEM. *P < 0.05, P < 0.01, NS, not significant.

**Figure 4** Cytological appearance of cultured neutrophils. Neutrophils (10⁶/ml) were cultured for 1 day in the absence (A, C) and presence (B, D) of sputum extracts. Cytospin preparations were subjected to Diff-Quick staining (A, B) and TUNEL (C, D) as described in ‘Materials and methods’. Arrows in A and B indicate cells with morphological features of apoptosis, and arrow heads in C those positive for TUNEL (magnification × 1000).
Discussion

We demonstrated here for the first time that a neutrophil survival-enhancing activity due to a reduction of apoptosis is present in the sputum of patients with DPB, and that this activity is attributable, at least in part, to GM-CSF but not to G-CSF and LPS. There is increasing evidence by a number of in vitro studies that several inflammatory mediators, including IL-1β, IL-8, IL-15, IFN-γ, G-CSF, GM-CSF, C5a, platelet-activating factor and leukotriene B₄ (LTB₄), suppress neutrophil apoptosis, thus enhancing its survival. The effects of IL-6 and TNFα on neutrophil survival are variable.15–18 In sputum and bronchoalveolar lavage fluid (BALF) of patients with DPB a high concentration of IL-1β, TNFα, IL-8 and LTB₄ are found.2 The bronchiolar epithelial cells in patients with DPB have been demonstrated, by immunohistochemistry on lung biopsy tissues, to strongly express GM-CSF,26 although the content of GM-CSF has not been reported. P. aeruginosa, which consistently colonizes the airways of DPB, has been shown to stimulate bronchial epithelial cells to release neutrophil survival-enhancing activity that is neutralized by anti-GM-CSF and anti-G-CSF antibody.27 The sputum studied here from which P. aeruginosa was isolated would be rich in LPS, which has a potential to enhance neutrophil survival.16 Therefore, we focused on GM-CSF, G-CSF and LPS as candidates of the neutrophil survival-enhancing activity in the sputum. Since the activity in the sputum was heat-labile and partially neutralized by anti-GM-CSF, this activity might be attributed to GM-CSF and other proteins such as IL-1β, IL-8, TNFα and C5a. Although we measured the GM-CSF content in sputum extracts by enzyme-linked immunosorbent assay (ELISA) in preliminary experiments, the concentration in all samples was lower than 10 pg/ml, which is the sensitivity of ELISA kits (R&D System Inc.) we use (data not shown). Nevertheless, we assume that GM-CSF was present in the sputum of DPB patients because GM-CSF has been demonstrated to be able to enhance neutrophil survival even at concentrations as low as 5 pg/ml,28,29 and because the antibody specific for GM-CSF indeed significantly reduced the neutrophil survival enhanced by the sputum extracts as well that by exogenous GM-CSF. The possibility cannot be ruled out that inhibitory factors against ELISAs such as proteases were present in the sputum extracts.30

Given that GM-CSF is present in the sputum of DPB patients, epithelial cells, neutrophils and macrophages in the airways could be sources of GM-CSF. Neutrophils to synthesize GM-CSF in vivo has been shown by the observation that neutrophils isolated from peripheral blood released the molecule after stimulation with calcium ionophore and phorbol 12-myristate 13-acetate (PMA).31 Macrophages and bronchial epithelial cells can produce GM-CSF upon stimulation with TNF, IL-1 and LPS found in the airways of DPB. Neutrophil elastase, likely to be abundant in the inflamed airways of DPB patients, is a potent inducer of GM-CSF in epithelial cells.33 Furthermore, bacterial products from P. aeruginosa, which was consistently isolated from the sputum of the DPB patients studied here, such as rhamnolipids and mucoid exopolysaccharide, have been demonstrated to stimulate epithelial cells to synthesize and release GM-CSF.33 A variety of these molecules at the inflammatory site of DPB could enhance the local production of GM-CSF, which might be responsible for the neutrophil survival-enhancing activity in sputum. Another possibility for the involvement of GM-CSF in the neutrophil survival enhancement by the sputum extracts is autocrine/paracrine mechanisms of GM-CSF by neutrophils during the culture with the sputum extracts.
Although the physiological stimuli for GM-CSF production by neutrophils have not been identified, the sputum of DPB patients is rich in proinflammatory cytokines and complements capable of activating neutrophils. Such mechanisms are under investigation.

Neutrophils are the primary effector cells in the acute inflammatory response triggered by microbes or irritants. They are activated to exert their biologic function and undergo necrosis, with their disintegration leading to the inevitable disgorge-ment of large quantities of histotoxic mediators into normal host tissues. For the limitation of tissue damage and restitution of tissue homeostasis, the acute inflammation is finally resolved, where apoptosis plays an important role in the clearance of neutrophils from the inflammatory site. Alternatively, the failure to induce apoptosis or the inefficient removal of apoptotic neutrophils would cause neutrophils to undergo necrosis, which would favor persistent inflammation. This is supported by a recent study using caspase-1-deficient mice in which the lipopolysaccharide-induced inflammatory response in lung was prolonged in association with impaired neutrophil apoptosis. 34 To our knowledge, the present study is the first report showing the presence of inflammatory cell survival-enhancing activity in chronic inflammatory airway diseases except for bronchial asthma. In the sputum of asthmatics, eosinophil survival-enhancing activity was detected and reduced by anti-GM-CSF and anti-IL-5 antibody. 22 Delayed eosinophil apoptosis induced by GM-CSF and IL-5 appears to contribute to the persistency of eosinophilic inflammation in the airways that occurs in asthma. 35,36 As in bronchial asthma, our results strongly suggest that the presence of neutrophil survival-enhancing activity might be one of the underlying mechanisms of the chronic inflammatory environment in the airways of patients with DPB and, by extension, CF.

Recently, long-term, low-dose macrolide treatment has been demonstrated to be effective in improving the survival rate of patients with DPB 37 and the respiratory function in those with CF. 38 Anti-inflammatory effects, including the inhibition of GM-CSF production from monocytes, lung fibroblasts and bronchial epithelial cells 39 and the acceleration of neutrophil apoptosis, 40 have been reported as the mechanisms of action of macrolide. 41 The apoptotic action of macrolides was also suggested by the observation that the DNA of neutrophils cultured with sputum from a DPB patient treated with erythromycin was fragmented (unpublished observation). These findings support the idea that the prolongation of neutrophil survival is involved in the pathogenesis in DPB.

In summary, a neutrophil survival-enhancing activity, partially due to GM-CSF, was detected in the sputum from patients with DPB who show the characteristic chronic neutrophilic inflammation. Our results suggest that delayed neutrophil apoptosis is involved in the pathogenesis of DPB and CF. Further investigation of other factors responsible for this activity and the regulatory mechanisms for the expression of these factors, especially in correlation with P. aeruginosa infection, could lead to the development of new therapeutic strategies for reducing the airway inflammation in DPB and CF.

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**References**