ORIGINAL RESEARCH LETTER

Peripheral alveolar nitric oxide concentration reflects alveolar inflammation in autoimmune pulmonary alveolar proteinosis

To the Editor:

Autoimmune pulmonary alveolar proteinosis (aPAP) is caused by an excess of autoantibodies against granulocyte-macrophage colony-stimulating factor (GM-CSF). In this situation, the clearance of intracellular debris in alveolar macrophages is impaired, and the alveolar spaces are occupied by excessive surfactant sediments. Several serum markers (lactate dehydrogenase (LDH), KL-6, carcinoembryonic antigen and surfactant protein D), alveolar–arterial oxygen difference ($P_{A-a}O_2$) and the percentage diffusing capacity of the lung for carbon monoxide (%*D*LCO) have a correlation with the disease severity and are used to evaluate the severity of aPAP [1].

Nitric oxide (NO) in expired gas is produced mainly by lung-resident cells, including epithelial cells and alveolar macrophages, which control the intracellular upregulation of inducible nitric oxide synthase (iNOS) under the influence of various types of inflammation. Exhaled nitric oxide fraction measured at a flow rate of 50 $mL \cdot s^{-1}$ (*F*ENO50; ppb) and peripheral alveolar NO concentration (CANO; ppb) measured and calculated using the two-compartment model method reflect NO production in the central airway and peripheral alveolar spaces, respectively [2]. The usefulness of *F*ENO50 is established for the management of central airway inflammation such as bronchial asthma and chronic obstructive pulmonary disease with eosinophilic inflammation [3, 4]; however, the usefulness of CANO is still unknown.

The polarity of activated macrophages in disease is generally divided into pro-inflammatory (so-called M1) and anti-inflammatory (so-called M2) types [5]. GM-CSF is considered to belong to the pro-inflammatory axis inducer of macrophage differentiation and the expression of the iNOS gene is generally observed in pro-inflammatory macrophages [6, 7]. Accordingly, we hypothesised that the measurements of CANO could reveal parts of the pathogenesis of aPAP and could be a useful marker for evaluating the degree of macrophage inflammation in aPAP patients.

10 aPAP patients diagnosed according to diagnostic criteria were registered consecutively from January 2014 to January 2017 [1]. Control subjects (n=6) were selected from persons presenting slight interstitial changes in chest radiography and/or computed tomography (CT) upon annual health check-ups. There were no symptoms and the examinations were undertaken to exclude active respiratory disease. Bronchoalveolar lavage fluid (BALF) results of the control subjects were within normal limits, including cell fraction and cytological and bacteriological examinations. The rate of reduction in forced vital capacity was <5% per year and the fibrotic area was <1% of the total lung area in CT images in the control subjects. None of the aPAP patients and control subjects had allergies under the treatment and none used immunosuppressive or antifibrotic drugs. The smoking ratios were no different between the aPAP and control group (40.0% *versus* 33.3%, respectively). Alveolar macrophages of aPAP patients were collected from BALF or whole-lung lavage (WLL) samples. In the WLL cases, the second and third lavage was used for the analysis to avoid contamination by cells derived from the central airway. The ratios of macrophages in the cells collected from WLL and BALF were >90% in all aPAP and control subjects. *F*ENO50 and CANO levels were measured using a chemiluminescence-based nitrogen oxide analyser (NA-723N; Chest, Tokyo, Japan), according to previous reports [8]. A threshold for CANO of 5.3 ppb was adopted based on previous

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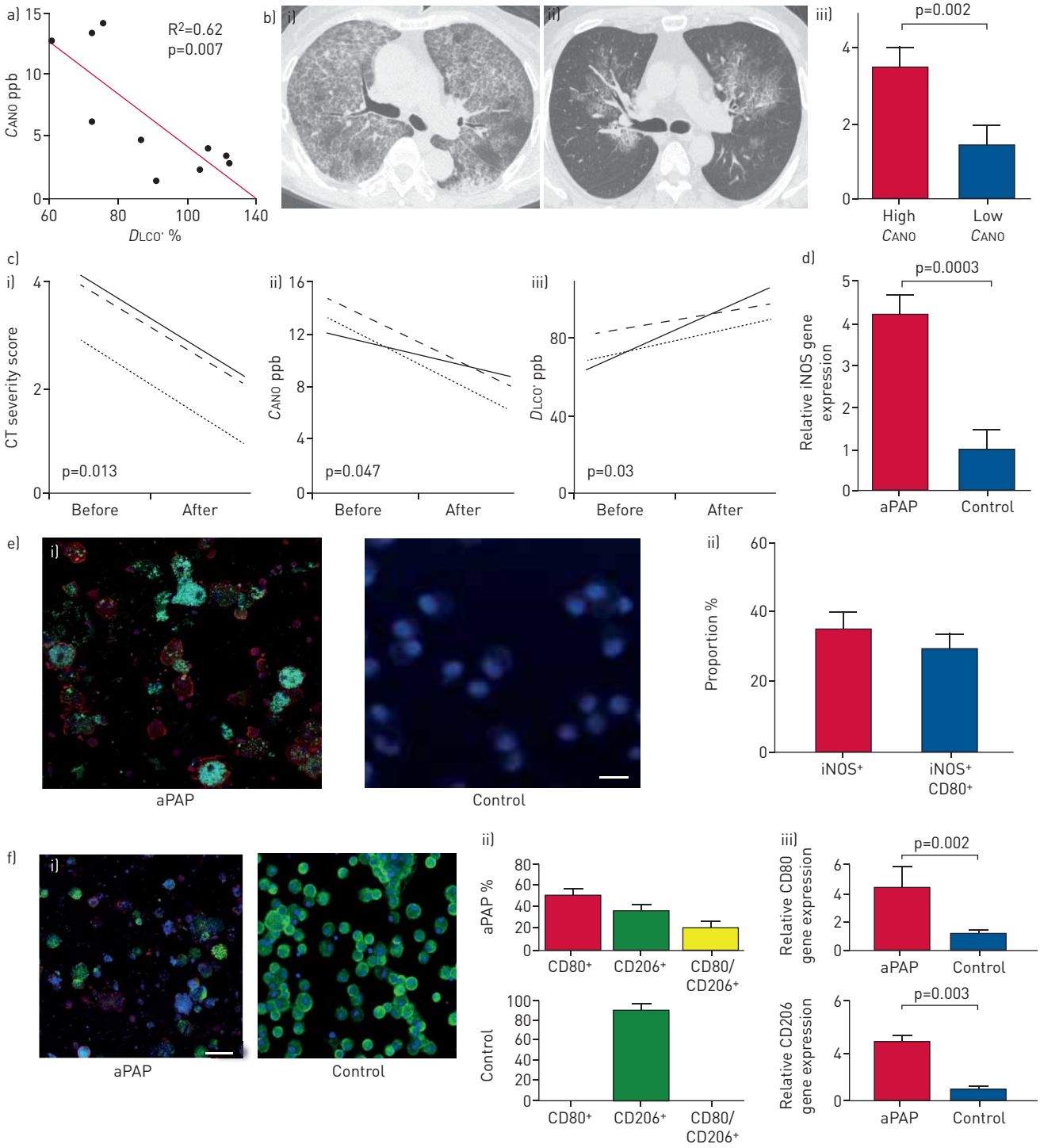

FIGURE 1 Nitric oxide synthesised from alveolar macrophages reflects disease severity, and peripheral alveolar nitric oxide concentration (C_{ANO}) could be a noninvasive and useful marker in the management of autoimmune pulmonary alveolar proteinosis (aPAP) patients. a) C_{ANO} has an inverse correlation with the percentage diffusing capacity of the lung for carbon monoxide calculated using alveolar volume measured by the helium dilution method (%D_{LCO}') in aPAP patients. b) Representative i), ii) computed tomography (CT) images and iii) CT severity scores of aPAP patients divided into i) high (⩾5.3 ppb) and ii) low (⩽5.3 ppb) C_{ANO} groups. c) i) CT severity scores and the values of ii) C_{ANO} and iii) %D_{LCO}' before and after whole-lung lavage (WLL), for individual aPAP patients (n=3). d) The relative expression of inducible nitric oxide synthase (iNOS) gene to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) gene in cells collected from bronchoalveolar lavage (BAL) and WLL in aPAP patients and control subjects. These values were measured using quantitative (q)PCR. e) i) Representative immunostaining images of cells from BAL and WLL in aPAP patients and control subjects with specific iNOS (green), CD80 (red) and 4′,6-diamidino-2-phenylindole (DAPI) (blue) antibodies. Scale bar=20 µm. ii) The ratio of iNOS-positive and double-positive (iNOS and CD80) in DAPI-positive cells from BAL and WLL in aPAP patients (n=3). f) i) Representative immunostaining images using specific CD80 (red), CD206 (green) and DAPI (blue) antibodies, scale bar=20 µm; ii) ratios of CD80-positive, CD206-positive and CD80/CD206 double-positive cells to DAPI-positive cells in aPAP patients and control subjects; iii) relative expression of CD80 and CD206 genes to GAPDH gene in the cells collected from BAL and WLL in aPAP patients and control subjects measured using qPCR. Data are presented as mean±SE.

studies [2, 9, 10]. The methods for evaluating the CT severity score and molecular biological analysis were performed in accordance with previous studies [11, 12]. For quantitative PCR, SYBR reagent (4367659; Thermo Fisher Scientific, Waltham, MA, USA) and δ-δ methods using glyceraldehyde 3-phosphate dehydrogenase (GAPDH) as the control were adopted. For immune staining, antibodies against iNOS (ab15123; Abcam, Cambridge, UK), CD80 (AF140; R&D Systems, Minneapolis, MN, USA), CD206 (ab64693; Abcam) and 4′,6-diamidino-2-phenylindole (D1306; Thermo Fisher Scientific) were used. For the statistical analyses, unpaired two-tailed t-tests and Pearson's analysis with JMP software (SAS Institute, Cary, NC, USA) were used. p-values <0.05 were considered to be statistically significant. Data are presented as mean±SE.

*Fe*NO50 did not show a correlation with %*D*LCO′ (based on alveolar volume measured by helium dilution method) and the serum markers (KL-6 and LDH), while *C*ANO showed a correlation with %*D*LCO′ (figure 1a) and the serum markers (data not shown). Next, aPAP patients were classified into high (>5.3 ppb; n=4) and low (⩽5.3 ppb; n=6) *C*ANO groups. The CT severity scores were significantly higher in the high *C*ANO group (figure 1b). To investigate the relationships between *C*ANO, CT severity scores, %*D*LCO′ and treatment, we collected the data of patients (n=3) whose disease was improved by WLL. All data before and after were measured within 6 months of receiving WLL. The values of *C*ANO, CT severity scores and %*D*LCO′ were improved after WLL in all cases (figure 1c). The relative expression levels of the iNOS gene to the GAPDH gene in the cells collected from patient bronchoalveolar lavage (n=3) were significantly higher compared with those of the control subjects (n=6) (p=0.0003) (figure 1d). In the immune-staining experiment using specific antibodies, iNOS-positive macrophages were found to account for 34.8±3.4% of the cells in the aPAP patients. However, no positive cells were observed in the control patients. Furthermore, 83±2.6% of the iNOS-positive cells were positive for CD80 (pro-inflammatory marker) (figure 1e).

Next, the macrophages were double-stained with anti-CD80 and anti-CD206 (anti-inflammatory marker) antibodies. CD80 single-positive cells (51.2±3.6%), CD206 single-positive cells (31.2±4.6%) and double-positive cells (CD80, CD206) (19.5±2.3%) were observed in the aPAP patients. However, only CD206 single-positive cells (92.1±1.1%) were observed in the control patients. Interestingly, higher mRNA expression levels of both CD80 and CD206 were observed in the aPAP patients compared with control subjects (figure 1f).

These results show that the values of *C*ANO from severe aPAP patients were much higher than in control subjects. Moreover, the *C*ANO levels in aPAP patients had significant correlations with the %*D*LCO′, KL-6 and LDH, and the reduction in *C*ANO occurred in parallel with improvements in the CT images after WLL [11]. Our results suggest that *C*ANO could be a useful marker for managing aPAP. In addition, our data suggest that alveolar macrophages are an important source of NO when the high expression of iNOS is upregulated by various stimulations. The upregulation of iNOS in alveolar macrophages is considered to be an indicator of pro-inflammatory activity and our results demonstrate that pro-inflammatory CD80-positive cells mainly produced NO. Generally, stimulation with human GM-CSF induces pro-inflammatory alveolar macrophages [6]. However, our data demonstrate that activated alveolar macrophages have both pro- and anti-inflammatory characteristics under the presence of the auto-GM-CSF antibody. Most alveolar macrophages expressed CD206 in control subjects, but the degree of activation of these cells was relatively low compared to those of the aPAP patients. Previous reports have suggested that the alveolar macrophages of GM-CSF knockout mice were activated with the upregulation of both pro- and anti-inflammatory markers [13]. It may suggest a compensatory mechanism to severe inflammation or an unmatured phenotype in the alveolar macrophages of aPAP. Several studies have suggested that GM-CSF is a type of "pro-inflammatory cytokine" for some diseases, including rheumatoid arthritis [14]. These insights may suggest that the auto GM-CSF antibody could have protective effects against "GM-CSF inflammation" in aPAP patients [15].

This study has limitations. The number of samples is small because the prevalence rate of aPAP is rare (<0.001% in Japan) [1]. The existence of two types of alveolar macrophages derived from BALF and WLL and previous WLL may have potential biases. It is possible that the existence of more macrophages in lungs of aPAP patients and/or NO from the alveolar epithelial cells affected our results. However, our results suggest that macrophage inflammation is essential for the patho-aetiology of aPAP and *C*ANO could be a noninvasive and useful marker for aPAP. Further prospective observations will complement our data in the future.

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