Smoking is associated with increased levels of extracellular peptidylarginine deiminase 2 (PAD2) in the lungs

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ABSTRACT

Objective. *Smoking is a well-established risk factor in rheumatoid arthritis (RA), and citrullination of self-antigens plays a pathogenic role in the majority of patients. Increased numbers of peptidylarginine deiminase 2 (PAD2)-containing macrophages have been demonstrated in bronchoalveolar lavage (BAL) fluid from smokers, but intracellularly located PAD cannot be responsible for citrullination of extracellular self-antigens. We aimed to establish a link between smoking and extracellular PAD2 in the lungs.*

Methods. *BAL fluid samples were obtained from 13 smokers and 11 non-smoking controls. Total protein content and C-reactive protein (CRP) concentration were determined after separating cells from the samples. PAD2 content in cell-free BAL fluids was measured by means of a PAD2-specific sandwich ELISA.*

Results. *Significantly increased levels of soluble PAD2 were detected in cell-free BAL fluids from smokers as compared to non-smokers (p=0.018). The PAD2 content correlated with the overall CRP levels (p=0.009) and cell count (p=0.016).*

Conclusion. *This first demonstration of increased levels of extracellular PAD2 in the lungs of smokers supports the hypothesis that smoking promotes extracellular citrullination of proteins. This may represent a pathological event upstream for the production of anti-citrullinated protein antibodies (ACPAs) among RA patients carrying HLA-molecules capable of binding citrullinated self-peptides.*

Introduction

Tobacco smoking remains a great health burden on modern society. Increased cell infiltration and intensified release of pro-inflammatory cytokines are observed in the lungs of smokers (1, 2). Smoking is a well-established risk factor for development of various autoimmune disorders, including rheumatoid arthritis (RA) (3, 4), in addition to other inflammatory diseases and cancer. The link between smoking and increased susceptibility to development of RA has been connected with increased citrullination in the lungs (5). Citrullination refers to the conversion of arginine residues to citrulline residues, a post-translational process catalysed by an enzyme family called peptidylarginine deiminases (PADs) (6). In humans five PADs, with different substrate specificities, exist (PAD 1-4 and PAD6) (7), and presence of PAD2 and PAD4 has been demonstrated in bronchial mucosal biopsy specimens and bronchoalveolar lavage (BAL) cells in both healthy smokers and non-smokers (8).

PAD2 and PAD4 are expressed in the inflamed synovium of RA patients suggesting the involvement of these two isotypes in the pathogenesis (6, 9). Around 80% of RA patients carry HLA-molecules containing the so-called 'shared epitope' motif capable of binding citrullinated self-peptides (10). A similar, and greatly overlapping, proportion of patients produce anti-citrullinated protein antibodies (ACPAs), a highly specific prognostic marker for RA (11). A recent study demonstrated lung abnormalities and increased levels of citrullinated proteins in the lungs of ACPA-positive RA patients, compared to ACPA-negative patients (12). In the same study, high levels of ACPAs were found in BAL fluid samples, suggesting that local autoantibody production occurs in the lungs (12). Shared citrullinated peptide sequences have been identified in the lungs and joints of RA patients, which further supports a link between the two anatomical compartments (13).

Extracellular PAD has not been quantified in the lung compartments. It is possible that established RA autoantigens, such as fibrinogen, are citrullinated extracellularly in the lungs of smokers, due to increased release of PADs. We have recently developed a sandwich ELISA specific for soluble PAD2 (14). Using this assay, we here test cell-free BAL fluid samples from 13 smokers and 11 non-smokers for content of extracellular PAD2.

Methods

Subjects

Patients undergoing surgery at the Department of Ear-Nose-Throat (ENT) surgery, Odense University Hospital,

were recruited. Patients with arthritis, asthma, allergy, rhinosinuitis, disseminated cancer, lung diseases (except COPD) and ex-smokers were excluded from the study. A total of thirteen smokers and eleven non-smokers were included. Patient characteristics are shown in Table I. The Scientific Ethical Committee of the Region of Southern Denmark approved the study, and all subjects signed a written informed consent. In relation to operation, both smokers and non-smokers underwent spirometry according to standard procedure. The FEV_1/FVC ratio was determined for each individual. Three smokers and two non-smokers failed to successfully undergo the spirometry analysis, *e.g.* due to laryngeal surgery.

Collection of bronchoalveolar lavage fluid

BAL fluid was collected during general anaesthesia. A flexible bronchoscope was placed in wedged position in the right middle lobe, and the lavage was performed with 3 x 50 mL sterile saline and aspirated under low pressure. Samples with iatrogenic bleeding were discarded. The samples were filtered through double layer cotton gauze. Cell viability was evaluated by trypan blue exclusion and total cell count was determined. Cells were separated by centrifugation for 10 min at 170 *g*. Cell-free BAL fluid protein concentrations were determined according to the Bradford method in a commercial Bio-Rad protein assay (Bio-Rad, Hercules, USA). C-reactive protein (CRP) was measured using CRP human singleplex bead kit (Life Technologies, CA, USA) on a Luminex platform. Supernatants were frozen at -80°C.

PAD2 measurements

The content of PAD2 in BAL supernatants was determined using a recently published PAD2-specific sandwich ELISA (14). In brief, ELISA plates were coated with anti-PAD2 monoclonal antibody (mAb) *DN2* (1 µg/mL), and BAL supernatants were diluted 1:4 in dilution buffer (PBS, 0.5% Tween-20, 2% bovine serum [Sigma], 20 µg/mL mouse IgG isotype control [Novus Biologicals; Cambridge, UK], pH 7.4). Biotinylated anti-PAD2 mAb *DN6* (1 µg/mL) was added, followed by incubation with streptavidin-conjugated horse radish peroxidise (Invitrogen; CA, USA) and development with o-phenylene-diamine substrate (Kem-En-Tec; Taastrup, Denmark). All standards and samples were measured in duplicates. Absolute PAD2 concentrations were calculated by regression analysis for the standard curve using four parameter logistic curve-fitting by means of the MARS software (BMG Labtech; Ortenberg, Germany).

Statistical analysis

The GraphPad Prism 5.0 (GraphPad Software, San Diego, USA) was used for statistical analyses. The D'Agostino-Pearson Omnibus test was used to determine whether data sets were normally distributed, in which case unpaired parametric *t*-tests were performed. Otherwise comparison between groups were made using Mann-Whitney U-test. Receiver operator characteristics (ROC) curves were applied to determine the ideal cut-off value, with respect to PAD2 positivity. A fisher's test was applied to determine associations between categorical variables. Spearman's rank correlation coefficient (r_s) was calculated to determine relationship between variables. The threshold value 0.20 ng/mL was assigned to all negative PAD2 measurements in all analyses. *P*-values <0.05 were considered significant.

Table I. Characteristics of subjects.

		Smokers Mean (± SEM)		Non-smokers Mean (± SEM)	*p*-value
Age (years)	n=13	53.77 (± 2.77)	n=11	61.64 (± 3.13)	0.07
Male/Female ratio	n=13	0.18	n=11	0.22	0.85
Package-years	n=13	26.08 (± 2.57)		NA	NA
Cell count (x10^6/mL)	n=13	10.16 (± 5.07)	n=10	4.04 (± 0.92)	0.42
Protein concentration (mg/mL)	n=13	0.23 (± 0.02)	n=11	0.19 (± 0.03)	0.18
CRP (pg/mL)	n=13	87.97 (± 48.6)	n=11	36.92 (± 13.3)	0.99
FEV_1/FVC (%)	n=10	70.2 (± 2.16)	n=9	73.89 (± 1.91)	0.22

CRP: C-reactive protein; FEV: Forced expiratory volume; FVC: Forced vital capacity.

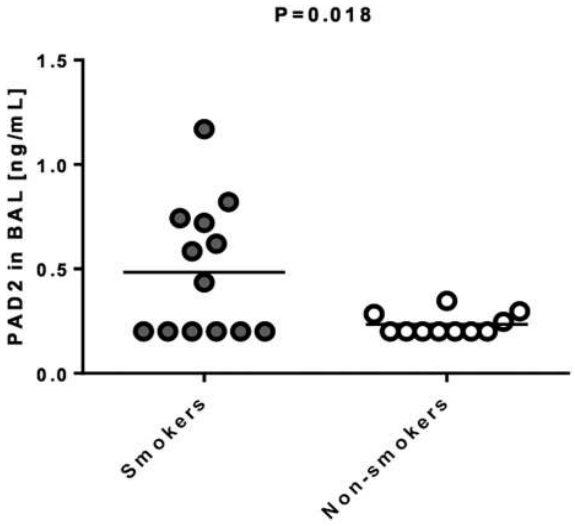

Fig. 1. PAD2 levels in BAL supernatants from smokers and non-smokers. Extracellular PAD2 was assessed in BAL fluids from 13 smokers (closed circles) and 11 non-smokers (open circles) by means of a PAD2-specific ELISA. The lower limit of detection was 0.2 ng/mL. The horizontal bars represent mean values

Results

BAL fluid samples from smokers had a PAD2 content of 0.48±0.09 ng/mL (mean ± SEM), while samples obtained from non-smokers contained 0.23±0.02 ng/mL PAD2 (*p*=0.018) (Fig. 1). Using a cut-off level of 0.39 ng/mL, as defined by ROC analysis, 7 out of 13 smokers were PAD2-positive *vs.* 0 out of 11 non-smokers (*p*=0.006). BAL fluid from smokers and non-smokers had similar total protein concentrations (Table I). Thus, an overall increase in protein concentration due to smoking could not explain the elevated PAD2 levels among smokers.

Table II. Correlation between parameters.

	PAD2 in BAL (ng/mL)	Cell count (x10^6/mL)	Protein concentration (mg/mL)	CRP (pg/mL)	FEV_1/FVC (%)
Cell count (x10^6/mL) (all patients)	r_s=0.50; p=0.016				
Protein conc. (mg/mL) (all patients)	r_s=0.21; p=0.33	r_s=0.28; p=0.20			
CRP (pg/mL) (all patients)	r_s=0.52; p=0.009	r_s=0.36; p=0.09	r_s=0.26; p=0.21		
FEV_1/FVC (%) (all patients)	r_s=-0.09; p=0.70	r_s=0.27; p=0.29	r_s=0.38; p=0.11	r_s=0.29; p=0.23	
Package-years (smokers only)	r_s=-0.15; p=0.62	r_s=0.38; p=0.20	r_s=0.38; p=0.20	r_s=0.05; p=0.87	r_s=0.44; p=0.20

PAD: peptidylarginine deiminase; BAL: Bronchoalveolar Lavage; CRP: C-reactive protein; FEV: Forced expiratory volume; FVC: Forced vital capacity.

There was an overall correlation between PAD2 concentration and cell-count in BAL, as shown in Table II. Within the individual groups, however, this positive correlation applied to non-smokers only (r_s=0.81, p=0.008 versus r_s=0.28, p=0.35 in smokers). Moreover, an overall correlation was observed between PAD2 levels and CRP levels (Table II). This correlation was borderline-significant within smokers (r_s=0.55, p=0.056), while a similar trend was observed among non-smokers (r_s=0.51, p=0.11). When smokers and non-smokers were grouped together, PAD2-positive BAL fluids (PAD2 > 0.2 ng/mL) contained markedly higher levels of CRP (p=0.004) and cell counts (p=0.003) than PAD2-negative BAL fluids (data not shown).

No further correlations were observed when analysing smokers and non-smokers separately. All analysed parameters were similar between PAD2-positive smokers and PAD2-negative smokers, except for significantly higher levels of CRP among the PAD2-positive subjects (p=0.01). No correlations were found between cell count, protein concentration, CRP, package-years and FEV_1/FVC ratio, as shown in Table II.

Discussion

According to a prevailing hypothesis, smoking disposes to ACPA-positive RA by increasing protein citrullination in the lungs (5). Intracellular citrullination by PAD may regulate *e.g.* release of neutrophil extracellular traps (NETs) and gene expression, which may promote tumour progression (15). On the other hand citrullinated extracellular proteins, such as fibrinogen, have been linked to the pathogenesis of RA (13). The existence of extracellular PAD, which may drive citrullination of extracellular proteins, has not been clearly demonstrated in the lungs. In the present study, we quantified soluble PAD2 in BAL fluid from smokers and non-smokers and found that smoking, indeed, was associated with increased extracellular PAD2 levels. Since the samples were cell-free, PAD2 must have been released either due to an active secretion, as has been demonstrated for mast cells (16), or as a result of leakage from dying cells, possibly damaged by chemicals contained in cigarette smoke (17).

In the group of smokers, 7 out of 13 subjects had relatively high PAD2 content in BAL fluid, compared to the 11 non-smokers. It is possible that smoking induces a rapid burst of extracellular PAD2, and that the enzyme has been cleared in subjects who had not smoked within a short time period before BAL. It is unknown to the authors when the subjects had their last smoke before the sampling.

We found no trend between package-years and PAD2 levels indicating that the increased content of extracellular PAD2 in smokers may indeed be an acute effect, and not be caused by the prolonged exposure of cigarette smoking. The PAD2 content did not correlate with the subjects' FEV_1/FVC ratio, indicating that COPD *per se* did not cause increased extracellular PAD2 levels. The PAD2 levels correlated with CRP levels indicating that an increased extracellular PAD2 content is linked to inflammation. This was supported by significantly higher levels of CRP among the PAD2-positive smokers than among PAD2-negative smokers.

Macrophages are the main producers of PAD2 during inflammation (6, 9) and account for around 90% of all cells in BAL (1, 8). Smoking elevates the overall content of cells in BAL fluid, mainly through increased numbers of macrophages (1), and cigarette smoke exposure has been shown to induce acute apoptosis of cultured alveolar macrophages (17). Our findings of free PAD2 in BAL fluid thus supplement those of Makrygiannakis *et al*., who demonstrated that smoking is associated with an increased expression of PAD2 enzyme in BAL cells and in bronchial mucosa (8). They further demonstrated that the expression of PAD4, which is mainly produced by neutrophils (6), was similar in the lungs of smokers and non-smokers.

Our study suffers from the low number of BAL samples available, and studies in larger cohorts are necessary to validate both the lack of correlations, as well as to confirm our positive findings. In conclusion, this is the first study to demonstrate a link between cigarette smoking, the most well-established environmental risk factor for RA, and extracellular PAD2 in the lungs. Increased levels of free PAD2 may be responsible for citrullination of extracellular proteins and possible break of tolerance towards these in the lungs of smokers who carry the shared epitope motif.

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