

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,

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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 FEV₁/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' FEV₁/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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