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Essential Role of Osteopontin in Smoking-Related Interstitial Lung Diseases

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Smoking-related interstitial lung diseases are characterized by the accumulation of macrophages and Langerhans cells, and fibrotic remodeling, which are linked to osteopontin (OPN) expression. Therefore, OPN levels were investigated in bronchoalveolar lavage (BAL) cells in 11 patients with pulmonary Langerhans cell histiocytosis (PLCH), 15 patients with desquamative interstitial pneumonitis (DIP), 10 patients with idiopathic pulmonary fibrosis, 5 patients with sarcoidosis, 13 otherwise healthy smokers, and 19 non-smoking controls. Furthermore, OPN overexpression was examined in rat lungs using adenoviral gene transfer. We found that BAL cells from patients with either PLCH or DIP spontaneously produced abundant amounts of OPN. BAL cells from healthy smokers produced 15-fold less OPN, and those cells from non-smoking healthy volunteers produced no OPN. BAL cells from patients with either idiopathic pulmonary fibrosis or sarcoidosis produced significantly less OPN, as compared with patients with PLCH. These data were confirmed by immunohistochemistry. Nicotine stimulation increased production of both OPN and granulocyte-macrophage colony stimulating factor by alveolar macrophages from smokers. Nicotinic acetylcholine receptor expression resembled the pattern of spontaneous OPN production and was dramatically increased in both PLCH and

DIP. OPN overexpression in rat lungs induced lesions similar to PLCH with marked alveolar and interstitial accumulation of Langerhans cells. Our findings suggest a pathogenetic role of increased OPN production in both PLCH and DIP by promoting the accumulation of macrophages and Langerhans cells. (Am J Pathol 2009, 174:1683–1691; DOI: 10.2353/ajpath.2009.080689)

Cigarette smoke is linked to a variety of lung diseases including chronic obstructive pulmonary disease, lung cancer, and interstitial lung diseases. Respiratory bronchiolar interstitial lung disease, desquamative interstitial pneumonitis (DIP), and pulmonary Langerhans cell histiocytosis (PLCH) belong to the group of smoking-related interstitial lung diseases.^{1–3} Cigarette smoke is a complex mixture of more than 4000 compounds and is known to cause systemic and pulmonary effects.⁴ However, the underlying mechanisms as to how cigarette smoking leads to the changes observed in smoking-related interstitial lung diseases are largely unknown.^{1–3}

Cigarette smoke induces inflammation, oxidative stress, and tissue injury, and has an important effect on the number, distribution, and activation state of macrophages and Langerhans cells.^{5,6} There is a strong epidemiological link between PLCH and smoking. PLCH is characterized by the accumulation of activated Langerhans cells originating from the distal bronchiole walls.^{1–3,7} The accumulations of Langerhans cells are poorly demarcated and extend to the adjacent alveoli, which often contain an abundance of pigmented macrophages. These areas show morphological changes similar to DIP.^{7,8} In DIP, the predominant feature is the accumulation of alveolar macrophages, densely filling the alveolar lumen, combined with moderate fibrotic interstitial remodeling.^{1,2}

As measured by bronchoalveolar lavage (BAL) in healthy individuals, cigarette smoking induces a 5- to 10-fold increase in alveolar macrophages in a dose-re-

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sponse curve.^{9–11} It was shown that concentrations of granulocyte-macrophage colony stimulating factor (GMCSF) in patients with PLCH are increased,¹² but the mechanisms that lead to the expansion of the pulmonary macrophage pool and fibrosis in smokers are poorly understood.^{1–3} Based on the findings of a microarray study, Woodruff et al¹³ have recently proposed that alveolar macrophages from smokers exhibit a distinctive macrophage activation state that is accompanied by increased OPN expression. Osteopontin is a glycoprotein found in the extracellular matrix of bone.¹⁴ However, multiple studies have reported cytokine properties of OPN in cell-mediated immunity.¹⁴ Further, OPN exhibits a strong chemotactic activity for macrophages, monocytes, Langerhans cells, and dendritic cells.^{15–17}

In the context of these findings we speculated that OPN might be involved in the pathogenesis of smoking-related lung interstitial diseases. We found abundant OPN production by alveolar macrophages from patients with PLCH and DIP. Alveolar macrophages from both healthy smokers and patients with DIP and PLCH show up-regulated nicotine receptor expression as a sign of chronic nicotine stimulation. Further, nicotine directly induced OPN and GMCSF in alveolar macrophages. Our data provides evidence for a role of osteopontin in the pathogenesis of smoking-related interstitial lung diseases.

Materials and Methods

Subjects

Eleven patients with PLCH and 15 patients with DIP were included. All patients displayed a typical histology and immunohistochemistry, shown either by transbronchial or video-assisted thoracoscopic biopsy and were currently cigarette smoking. In addition, 10 non-smoking patients with idiopathic pulmonary fibrosis (IPF), diagnosed according to the ATS/ERS-criteria,¹⁸ and five non-smoking patients with histologically proven sarcoidosis served as a disease control. Thirty-two healthy volunteers were included as a control group, who were further divided in 13 smokers and 19 never-smokers. The study was approved by the local ethics committee of the University of Freiburg.

Preparation of BAL-Derived Cells

Bronchoscopy and BAL were performed using a standard technique. In brief, 300 ml of sterile saline (0.9% NaCl) were instilled into the middle-lobe segment in 20 ml aliquots. Every aliquot was recovered immediately by gentle hand suction.

Stimulation of BAL Cells with Nicotine

BAL cells were processed as previously described.¹⁹ In brief, freshly isolated lavage cells were washed three times with PBS (Dulbecco,) and then cultured at a density of 1×10^6 cells/ml over a period of 24 hours in RPMI-1640 (Gibco, Eggenstein, Germany), supplemented with

2% heat inactivated human serum, 100 U/ml penicillin, and 100 μ g streptomycin in 24-well tissue culture plates in a 5% CO₂ humidified atmosphere at 37°C. The viability of the cells was determined by Trypan blue dye exclusion and always exceeded 80%. Nicotine [1-methyl-2-(3-pyridyl)pyrrolidine] 10^{-4} , 10^{-5} , 10^{-6} M/L, D-tubocurarine 10 μ mol/L, α -bungarotoxin 10 μ mol/L, polyhydroxyakanoate, 5 μ g/ml, phorbol 12 myristate 13 acetate, 10^{-8} M/L, or 1 μ g/ml lipopolysaccharide (all from Sigma, Deisenhofen, Germany) were added to the culture medium as needed. At the end of the culture period cell-free supernatants and cell pellets were harvested and stored at -70°C until assayed for OPN concentrations and reverse transcription (RT)-PCR. Nicotine was tested for potential endotoxin contamination by use of the Endotox assay (Sigma, Deisenhofen, Germany).

BAL Cell Differential Count and Cell Culture

Differential cell counts were determined by enumerating at least 200 cells at a cell smear stained by May-Grunwald staining.

Enzyme-Linked Immunosorbent Assay

Osteopontin was quantified using the following enzyme-linked immunosorbent assay (ELISA) kit: human OPN (IBL, Hamburg, Germany). The detection limit for OPN ELISA was 5 ng/ml. GMCSF was detected by a commercially available ELISA system (R&D, Wiesbaden, Germany). For duplicate samples an intra-assay coefficient of variation of <10% were accepted and interassay coefficient of variation of <20% were accepted. All presented values are not adjusted for BAL cell differentials. OPN concentrations below the detection limit of 5 ng/ml were held to be 0 ng/ml.

Lung Tissue Specimens

Lung wedge biopsies were obtained from five subjects with PLCH and five subjects with DIP by video-assisted thoracic surgery. Furthermore, normal lung tissue was obtained from five smokers and five non-smokers who underwent lobectomy due to a benign lung tumor. None of the patients were taking antibiotics or immunosuppressants at the time of surgery.

Reverse Transcription and Real Time PCR

Total RNA was extracted from 1×10^6 cells using TRIzol Reagent according to the manufacturer's protocol (Invitrogen, Karlsruhe, Germany). Total RNA was reverse-transcribed with StrataScript RT (Stratagene, CA) using oligo (dT)_{12–18} primer to produce cDNA according to the manufacturer's protocol. Specific primers for human OPN, nicotinic acetylcholine receptor subunits, and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) were designed using Primer3 software (Whitehead Institute for Biomedical Research, Cambridge; <http://www.broad>.

Table 1. Primers Used in RT-PCR-Analysis

Gene product	Forward primer	Reverse primer
Osteopontin	5'-AAGAAGTTTCGACACCTGACATC-3'	5'-GATGGCCTTGTATGCACCATTC-3'
b2 AChR	5'-TCCCTTCCAAACACATCTGG-3'	5'-TGCTGCCATCATAGGAGACC-3'
α 3 AChR	5'-ATGCTGTGCTGTCCCTCTCTG-3'	5'-GCAGGGTGAAAACCCACAGAA-3'
α 4 AChR	5'-GGTCGGGACCACTTGTGTA-3'	5'-ATCTAGAGCCCGCAGGTG-3'
α 6 AChR	5'-CGACCTGTACGGTACACT-3'	5'-ATGTCGGGCTTCCAAATCTT-3'
α 7 AChR	5'-TACTTCTCCCTGAGCCTCCTG-3'	5'-TTTCCAAATCTGGCCATCTGG-3'
GAPDH	5'-CACCAGGGCTGCTTTTAACT-3'	5'-GATCTCGCTCCTGGAAGATG-3'

AChR: acetylcholine receptor.

mit.edu/genome_software/other/primer3.html), Amplify1.2 software (University of Wisconsin; <http://engels.genetics.wisc.edu/amplify>) and using LocusLink and GenBank databases (National Center for Biotechnology Information; <http://www.ncbi.nlm.nih.gov/LocusLink/index.html>). All primers were intron-spanning (Table 1) and synthesized by MWG-Biotech (MWG-Biotech AG, Ebersberg, Germany). Real-time PCR was performed using the iQ SYBR Green SuperMix, iCycler thermocycler, and iCycler iQ 3.0 software (Bio-Rad Laboratories, Munich, Germany) according to the manufacturer's protocol. A threshold cycle value (Ct) was calculated and used to calculate the relative level of OPN and nicotinic acetylcholine receptor (nAChR) subunits mRNA by the following formula: $2^{(Ct \text{ GAPDH} - Ct \text{ Target})} \times 10,000$ for each cDNA sample.

Immunohistochemistry

Immunohistochemistry using anti-human Osteopontin mAb (MPIIB10, isotype mouse IgG1; DSHB, University of Iowa) and a peroxidase-labeled streptavidin-biotin technique (DAKO LSAB2 System; DakoCytomation, Germany) was performed as previously described.¹⁹

Survival Analysis

Alveolar macrophages from five smokers were cultured in RPMI supplemented with 10% fetal calf serum in the presence of GMCSF (100 U/ml LeucomaxTM, Novartis Basel, Switzerland), osteopontin (500 ng/ml; Chemicon, Germany) and GMCSF combined with osteopontin. Following 4 days cell culture cell survival was calculated using Trypan blue dye extinction. All experiments were done in triplicate.

Animal Experiments with AdhOPN

Adenoviral vectors were used to examine the effect of transient overexpression of OPN on rat lungs. The construction of adenoviral vectors is described in detail elsewhere.²⁰ Human OPN cDNA (in pGEM3Z vector, a gift from British Biotech Pharmaceuticals Ltd., Oxford, UK) was cloned into a shuttle vector with a human cytomegalovirus promoter and co-transfected on 293 cells with a plasmid containing E1- to E3-deleted type 5 adenoviral genomes. The resulting replication-deficient virus was amplified and purified by CsCl gradient centrifugation and PD-10 Sephadex chromatography, and finally

plaque titered on 293 cells. The vector was checked for integrity of viral DNA (by restriction digest and Southern blot) and expression of the desired protein (by ELISA). Control vectors (AdDL70) with no insert in the E1 region were produced in the same way.

Female Sprague-Dawley rats (Charles River Laboratories, Montreal, Quebec, Canada), weighing 200 to 250 g, were housed under special pathogen-free conditions. Rodent laboratory food and water was provided *ad libitum*. The animals were treated in accordance to the guidelines of the Canadian Council of Animal Care. All animal procedures were performed with inhalation anesthesia with isoflurane (MTC Pharmaceuticals, Cambridge, Ontario, Canada). A total of 1×10^9 plaque-forming units of AdhOPN or AdDL70 were administered intratracheally in a volume of 300 μ l PBS via intubation. AdhOPN-treated rats were sacrificed by abdominal aortic bleeding at days 2, 7, 14, and 21 ($n = 3$ per group). AdDL70-treated control animals were sacrificed at day 7 ($n = 3$). For histological examination, lungs were inflated and fixed by intratracheal instillation of 10% neutral buffered formalin at a constant pressure of 20 cm of water for 5 minutes. After fixation for 24 hours, lungs were paraffin-embedded, cut in 3- μ m sections, and stained with H&E. Immunohistochemistry was carried out with antibodies against S100 (Dako Cytomation, Hamburg, Germany) polyclonal rabbit Z 0311; CD1a monoklonal mouse, clone 010 (Dako); and CD68 monoklonal mouse, clone PG-M1 (Dako).

Statistical Analysis

Data are presented as box plots. Statistical comparisons between experimental and control data for the *in vitro* studies were made by analysis of variance with post hoc Fisher's protected least significant difference. Correlation was determined by a simple regression model. Data of binominal distribution were logarithmically transformed prior statistical analysis. Probability values were considered significant if they were less than 0.05.

Results

Patient data, total BAL cell counts and BAL cell differentials are listed in Table 2. There was a significant increase in BAL total cell counts and alveolar macrophage cell counts in patients with PLCH, DIP and smokers in contrast to non-smoking healthy individuals (all $P < 0.001$).

Table 2. Characteristics of Patients and Bronchoalveolar Lavage

	PLCH	DIP	Smoker	IPF	Sarc	Control
Age [years]	44 ± 11	54 ± 11	50 ± 19	71 ± 7	49 ± 9	32 ± 12
Sex [male/female]	7/4	11/4	8/5	9/1	2/3	7/12
Smoking history	11/11	15/15	13/13	7/10	0/5	0/19
Pack years	47 ± 10	46 ± 12	28 ± 15	10 ± 10*	0	0
BALF Cotinin $\mu\text{g/ml}$	25 ± 22	20 ± 14	19 ± 21	n.d.	n.d.	<2
BAL differential cell count						
BAL cell count/100 ml [10^6]	25 ± 12	44 ± 25	22 ± 12	16 ± 11	13 ± 8	10 ± 5
BAL macrophage count [10^6]	23 ± 10	38 ± 23	20 ± 12	12 ± 7	5 ± 2	9 ± 5
Macrophages [%]	93 ± 4	89 ± 21	91 ± 7	77 ± 12	48 ± 23	89 ± 7
Lymphocytes [%]	3 ± 2	7 ± 16	6 ± 5	10 ± 6	49 ± 22	9 ± 6
Neutrophils [%]	3 ± 3	3 ± 5	2 ± 5	9 ± 7	2 ± 1	1 ± 1
Eosinophils [%]	1 ± 1	1 ± 2	0 ± 0	3 ± 2	1 ± 1	0 ± 0

BAL: bronchoalveolar lavage; PLCH: pulmonary langerhans cell histiocytosis; DIP: desquamative interstitial pneumonia; IPF: idiopathic pulmonary fibrosis; Sarc: sarcoidosis. Data are means $\pm$ SD. *All IPF patients did not smoke at the time point of BAL, but 7 patients out of 10 had a history of cigarette smoking. N.D. represents not done.

BAL-Cells from Patients with Smoking-Related Interstitial Lung Diseases and IPF Spontaneously Produce High Amounts of OPN

BAL-cells from patients with smoking-related interstitial lung disease spontaneously produced up to 400 ng/ml OPN, while BAL-cells from healthy nonsmoking volunteers produced only marginal OPN levels near the detection limit of the ELISA system (Figure 1). In detail, BAL-cells from patients with PLCH produced significantly more OPN than BAL-cells from patients with DIP ($P < 0.001$). Spontaneous OPN production by BAL-cells from patients with PLCH or DIP was strongly increased compared with non smoking controls ($P < 0.0001$) and otherwise healthy smokers ($P < 0.0001$ compared with PLCH, $P = 0.03$ compared with DIP). There was also an increase in the spontaneous OPN production by BAL-cells from patients with IPF, but the increase was significantly lower than that associated with patients with PLCH. A further, statistically insignificant increase in spontaneous OPN production by BAL-cells from patients with sarcoidosis and regularly smoking healthy volunteers, was registered by comparison with healthy nonsmoking volunteers; neither stimulation with LPS nor with PMA and PHA significantly up-regulated OPN production in patients with PLCH and DIP and healthy volunteers (data not shown). In addition, there was a correlation

between OPN production by BAL-cells and total alveolar macrophage cell count in 100 ml BAL ($P < 0.0001$, $r = 0.64$).

OPN Concentration Is Enhanced in BALF in PLCH and DIP

To evaluate total pulmonary OPN production, we also tested OPN concentrations in BAL-fluids (BALF). Neither in BALF from non-smokers, nor from sarcoid patients were OPN levels above the detection limit of the ELISA system. In all BALF from patients with PLCH and DIP we detected OPN, but in low concentrations (PLCH: 10.3 ± 3.6 ng/ml, DIP: 8.2 ± 3.5 ng/ml; data not shown). In some of the BALF from patients with IPF (2.9 ± 3.9 ng/ml), and healthy smokers (3.3 ± 5.7 ng/ml), OPN was detectable. Because of the known instability of the protein during thawing and freezing processes, we did not attempt to concentrate BALF.

OPN Is Highly Expressed in Biopsies of Patients with Smoking-Related Interstitial Lung Diseases

Immunohistochemistry with anti OPN antibodies revealed strong OPN expression in lung biopsies from patients with PLCH (Figure 2D) compared with healthy controls (Figure 2E). OPN was also markedly expressed in specimens from patients with DIP but to a lower extent. In both PLCH and DIP a marked staining for OPN was observed in alveolar and infiltrating interstitial macrophages and to far lesser extent in alveolar epithelial cell (Figure 2, A–D). Furthermore, OPN expression was also found in alveolar macrophages from smokers (Figure 2F), while its expression in lung specimens of non-smoking individuals was very low or absent.

Nicotine Stimulation Increases OPN and GM-CSF Production from BAL-Cells of Smokers

To test if nicotine stimulates OPN production, alveolar macrophages derived from healthy smoking controls and nonsmoking controls were stimulated with nicotine in

Figure 1. Spontaneous OPN production of BAL-cells from patients with PLCH, DIP, IPF, sarcoidosis, smokers and healthy controls. Box plots: horizontal lines represent median, 25 and 75 percentiles, and small lines characterize 10 and 90 percentiles (* $P < 0.05$; ** $P < 0.001$ compared with control).

Figure 2. Localization of OPN-immunoreactive protein in the lung tissue biopsies obtained from patients with (PLCH), desquamative interstitial pneumonia (DIP), and controls. **A–F:** immunohistochemistry for OPN. **A** ($\times 200$ magnification) and **C** ($\times 50$ magnification): DIP; **B** ($\times 200$ magnification) and **D** ($\times 50$ magnification): PLCH; **E:** normal lung ($\times 200$ magnification, **Inset:** $\times 50$ magnification); **F:** current smoker ($\times 200$ magnification, **Inset:** $\times 50$ magnification). Weak OPN immunoreactivity (DIP) and strong OPN immunoreactivity (PLCH) can be seen within the interstitium (PLCH) and in the intraalveolar located cells (PLCH and DIP); and a weaker OPN immunoreactivity in alveolar macrophages from a current smoker. Control lung did not show any detectable signal (**E**).

varying concentrations with or without the presence of α -tubocurarine, a non-selective nicotinic acetylcholine receptor antagonist. In alveolar macrophages from active smokers we observed a significant increase in OPN production in response to nicotine (Figure 3A). This effect was inhibited by the non-selective antagonist α -tubocurarine. In contrast, nicotine stimulation did not induce OPN-production in alveolar macrophages derived from non-smoking controls. An identical pattern was seen in PCR analysis (data not shown). The selective antagonist for $\alpha 7$ nAChR subunit, bungarotoxin- α (data not shown), did not abrogate the effect of nicotine stimulation, suggesting that this receptor is not involved in the regulation of OPN expression. In addition to OPN, nicotine stimula-

tion also regulated GMCSF production by alveolar macrophages of smokers with an identical pattern as described for OPN (Figure 3B).

Alveolar Macrophages from Smokers, PLCH and DIP Have Up-Regulated nAChR Subunit Expression as a Sign for Chronic Nicotine Stimulation

Since several studies described that nicotine stimulation paradoxically increases its own receptor expression, we wondered if nicotine up-regulates the nAChR subunit expression by alveolar macrophages from non-smoking controls. Indeed, nicotine stimulation of alveolar macrophages increased all tested nAChR subtypes (Figure 4, A–D). Highest levels of mRNA expression were found for $\beta 2$ and $\alpha 7$ subunits (Figure 4, A and B).

Based on these findings, we wondered if BAL-cells from our patient cohort as well as from healthy smoker and nonsmoking controls express distinct levels of nAChR subunits. Alveolar macrophages from patients with PLCH and DIP expressed abundant levels of $\beta 2$ nAChR and $\alpha 7$ nAChR in contrast to nonsmoking individuals ($P < 0.0001$, Figure 4). Also the expression of $\alpha 3$ nAChR, $\alpha 4$ nAChR, and $\alpha 6$ nAChR were increased compared with nonsmoking controls. In addition, BAL-cells from smoker expressed increased nAChR subunits mRNA than nonsmoking controls.

Active Smokers Have Elevated Cotinine Levels in BALF

For analysis of the recent smoking behavior we analyzed cotinine concentrations, a metabolite of nicotine in BALF. Cotinine concentrations were detectable in all current smokers, while we could not detect any cotinine in the BALF of non-smokers above the detection limit of $2 \mu\text{g/ml}$ (Table 2). There was no statistically significant difference in cotinine concentrations among patients with smoking related interstitial lung diseases and healthy smokers.

Combined Stimulation with OPN and GMCSF Enhances the Survival of Normal Alveolar Macrophages of Nonsmoking Individuals

Since studies with dendritic cells and T-cells had suggested an influence of OPN stimulation for cell survival

Figure 3. Nicotine stimulation increases OPN and GMCSF production by BAL-cells. Increase in OPN (**A**) and GMCSF (**B**) protein production of BAL-cells from a smoker following a single stimulation with different doses of nicotine (10^{-4} to 10^{-6} M) with or without the nonselective nAChR antagonist α -tubocurarine ($10 \mu\text{mol/L}$). One representative experiment out of five with AM from five different smokers. $*P < 0.05$

Figure 4. Relative levels of nAChR subunit mRNA expression in alveolar macrophages from patients with PLCH, DIP, smokers and controls. **A:** relative levels of $\beta 2$ nAChR expression. **B:** relative levels of $\alpha 7$ nAChR expression. **C:** relative levels of $\alpha 3$ nAChR, $\alpha 4$ nAChR, $\alpha 6$ nAChR expression. **D:** increase in the relative levels of all tested nAChR subunits following nicotine stimulation by AM from a healthy volunteer. One triplicate experiment out of three with AM from three different healthy individuals. Box plots: horizontal lines represent median, 25th and 75th percentiles, and small lines characterize 10th and 90th percentiles (* $P < 0.05$; ** $P < 0.005$ compared with control).

we tested survival rates of alveolar macrophages derived from non-smoking individuals following stimulation with OPN and GMCSF. In contrast to GMCSF, stimulation with OPN alone did not alter the survival of alveolar macrophages following 4 days of cell culture. However, the combined stimulation with OPN and GMCSF significantly enhanced the survival of alveolar macrophages (Figure 5).

Adenoviral-Vector Mediated OPN Overexpression in Rat Lungs Results in Langerhans Cell and Macrophage Accumulation with Strong Similarities to Human PLCH

To examine if OPN is able to induce Langerhans cell and macrophage accumulation, we over-expressed OPN in rat lungs by adenoviral vectors. Efficient transfection of the OPN gene and production of human OPN was confirmed by ELISA of BALF. Transgenic hOPN in BALF was transient, peaked by day 7, and was no longer detectable by day 14 (Figure 6A). Histological analysis did show a marked accumulation of Langerhans cells and macrophages in rat lungs over-expressing hOPN (Figure 6, B–L), most prominent by day 7. Immunohistochemistry showed a marked increase in CD1a⁺ and S100⁺ Langerhans cells and a moderate increase in CD68⁺ macrophages (Figure 6, E–L).

Figure 5. Effect of OPN (500 ng/ml) and GMCSF (100 U/ml) on the survival of alveolar macrophages from smokers cultured for 96 hours. One representative experiment out of five with AM from five different smokers. * $P < 0.05$.

Discussion

Recent microarray studies suggested an increase of OPN, a glycoprotein with cytokine-like properties, and a specific activation status of alveolar macrophage of cigarette smokers.^{13,21} Smoking-related interstitial lung diseases are characterized by macrophage accumulation and fibrotic remodeling of the lung.^{1–3} There is a clear link between cigarette smoke and induction of these diseases, however the responsible substances in cigarette smoke need to be identified. Here we report that OPN is highly up-regulated in patients with smoking-related interstitial lung diseases and we provide evidence that nicotine induces OPN and GMCSF production by macrophages, which enhances cell survival. Our findings suggest that nicotine stimulation induced by cigarette smoking promotes macrophage accumulation and fibrotic lung tissue remodeling via an increase in OPN and GMCSF production.

Our data demonstrate an increase in spontaneous OPN-production by BAL-cells in smoking-related interstitial lung disease. BAL-cells from patients with PLCH produced the highest OPN levels spontaneously, while BAL-cells from patients with DIP produced slightly lower levels. In never-smoking, healthy volunteers, no spontaneous OPN production by BAL-cells was observed. Neither stimulation with LPS nor stimulation with PMA and PHA were able to induce OPN-production in BAL-cells derived from healthy, non-smoking volunteers. Alveolar macrophages from healthy smokers produced OPN in concentrations of approximately 10 ng/ml, which is 15-fold lower than the production of BAL-cells from patients with PLCH. OPN production by BAL-cells from patients with IPF was also increased, but less compared with patients with PLCH and DIP. In addition, OPN-production in sarcoidosis, a lymphocyte-rich inflammatory interstitial lung disease, was only marginally increased. Our data suggest that an increase in OPN-production is not a common finding of all inflammatory lung diseases, but is rather an indicator of a special type of macrophage activation. OPN concentrations in BALF reflected the find-

Figure 6. Adenoviral-vector mediated overexpression of hOPN induces lesions with strong similarities to human PLCH. **A:** effectiveness of gene transfer is demonstrated by hOPN ELISA. hOPN induced pulmonary lesions peaked at day 7. In the BALF of mice transfected with the control vector (AdL70) hOPN expression was absent. **B–D:** H&E staining of rat lungs after administration of AdhOPN. **B:** $\times 50$ magnification, **C:** $\times 200$ magnification. **D:** control vector $\times 50$ magnification. **E–L:** immunohistochemistry for CD1a. **E:** AdhOPN vector ($\times 50$ magnification), **F:** AdhOPN vector ($\times 200$ magnification), **G:** control vector ($\times 100$ magnification) and for S100. **H:** AdhOPN vector ($\times 50$ magnification), **I:** control vector ($\times 100$ magnification), and for CD68. **J:** AdhOPN vector ($\times 100$ magnification), **K:** AdhOPN vector ($\times 200$ magnification), **L:** control vector ($\times 100$ magnification).

ings of spontaneous production by BAL-cells *in vitro*. However, OPN levels in BALF were just above the detection limit of the ELISA system, and therefore have to be interpreted cautiously. Microarray studies already suggested increased OPN expression in smokers,^{13,21} but this is the first study showing a clear link between OPN and smoking-related interstitial lung disease.

Immunohistochemistry further confirmed the described *in vitro* data. OPN expression was highly increased in biopsies from patients with PLCH and DIP. In PLCH, particularly the infiltrates of macrophages and

CD1a⁺ cells highly expressed OPN. In DIP, the alveolar conglomerates of macrophages were strongly positive for OPN. Alveolar epithelial cells type II were positive for OPN in both diseases. Pardo et al²² have previously shown that in IPF, mainly alveolar epithelial cells type II produce OPN. However, our findings clearly indicate that in PLCH and DIP macrophages also contribute to the increase in OPN production. Recently, it was shown that alternative translation of a single OPN mRNA species generates a secreted and intracellular isoform.²³ The intracellular OPN isoform was not detected by our ELISA system and might explain why

immunohistochemistry data from other groups^{22,24} and OPN production measured by our ELISA system in sarcoidosis and IPF are slightly divergent.

For further analysis, we wondered which substance(s) in cigarette smoke might be responsible for inducing OPN. Since several articles recently described an immuno-regulatory role of nicotine,^{25–27} we tested if nicotine might influence OPN-production by alveolar macrophages. Therefore, we stimulated BAL-cells from healthy, currently smoking and nonsmoking individuals with nicotine. Interestingly, nicotine increased OPN synthesis only in BAL-cells from currently smoking individuals, but not in healthy nonsmoking volunteers. Similar data were obtained for GMCSF. One explanation for the discrepancy between BAL-cells derived from healthy nonsmokers and smokers could be that only alveolar macrophages from currently smoking individuals express functionally active nAChR. Indeed, several studies have shown that nicotine paradoxically up-regulates its own nicotinic acetylcholine receptors, and that repetitive stimulation with nicotine, also known as chronic nicotine stimulation, causes the expression of high levels of functionally active nicotinic acetylcholine receptors.^{28,29} This paradoxical effect of nicotine stimulation is a key mechanism in the evolution of cigarette smoke addiction.^{28,29} The mRNA expression of all five tested nAChR subunits was increased by nicotine stimulation in alveolar macrophages derived from nonsmoking healthy individuals.

Further analysis revealed that the mRNA expression of all tested nAChR subunits are abundantly up-regulated in alveolar macrophages from patients with PLCH and DIP. Alveolar macrophages from healthy smokers expressed significantly lower levels compared with alveolar macrophages from patients with PLCH or DIP, but clearly higher levels than seen in healthy nonsmokers. This is most likely caused by chronic nicotine stimulation on the basis of cigarette smoking. Lebargy et al³⁰ have reported that cigarette smoke can increase acetylcholine receptor binding sites in PBMCs. In addition, several studies reported that alveolar macrophages derived from smokers have an increase in p38 MAP kinase expression,^{31,32} which is induced by nicotine stimulation.³³ Interestingly, p38 MAP kinases also regulate osteopontin and GMCSF expression.^{34,35} In summary, these findings suggest that the altered activation status of alveolar macrophages from cigarette smokers, and particularly from patients with PLCH and DIP, is partly explainable by chronic nicotine stimulation.

Cigarette smoking induces a 5- to 10-fold increase in alveolar macrophages in healthy individuals.^{9–11} It is also well known, that alveolar macrophages from smokers disclose enhanced survival and reduced apoptosis rates.³⁶ In DIP and PLCH a substantial elevation of alveolar macrophage cell counts was noted. The underlying mechanisms for this increase are poorly understood. Tazi et al¹² showed an increase in GMCSF production by bronchial epithelial cells in patients with PLCH. Interestingly, nicotine stimulation of bronchial epithelial cells is able to increase their own GMSCF production.^{37,38} In our study we show that OPN, in combination with GMCSF, enhances survival of alveolar macrophages. This suggests that chronic nicotine stimulation might be involved

in this increase in OPN and GMCSF production. However, our findings do not explain why patients with PLCH have a 15-fold increase in OPN compared with healthy smokers. Clearly, there are additional, yet unknown, factors involved in the increased OPN and GMSCF production in smoking-related interstitial lung diseases.

There was a correlation between OPN and macrophage accumulation within the lung. In some patients the macrophage count was 10 times increased with a recovery of up to 70 million macrophages per 100 ml BAL. This might be related to the described effect of OPN on macrophage survival, but could also be caused by an increase in macrophage recruitment to the lung, and/or increased macrophage proliferation *in situ*. Several articles described chemotactic activity of OPN on macrophages.^{15,16} Therefore, increases in pulmonary OPN levels will lead to an increase in the influx of macrophages and will facilitate macrophages accumulation.^{17,39}

To further strengthen our hypothesis, we investigated the effects of increased presence of OPN in rat lungs. In rats, OPN was overexpressed by intratracheal administration of adenoviral vectors containing the full length gene for human OPN. This approach resulted in the transient presence of high intrapulmonary levels of OPN and caused substantial accumulation of Langerhans cells and macrophages in the alveolar lumen and interstitial space. The histological changes resembled the changes observed in human PLCH, strongly supporting our hypothesis of a fundamental role for OPN in smoking-related interstitial lung diseases.

In conclusion, our data show that OPN is highly up-regulated in patients with smoking-related interstitial lung diseases. We demonstrate that alveolar macrophages from patients with DIP and PLCH displayed the highest level of nAChR expression, indicating chronic nicotine stimulation. Nicotine itself up-regulates OPN and GMCSF production by alveolar macrophages, and OPN in concert with GMCSF enhances survival of alveolar macrophages. These data suggests an important role for OPN in smoking related interstitial lung disease. This hypothesis was further strengthened by overexpression of OPN in rat lungs in which induced pathological lesions closely resembling human PLCH. Thus, we identified a link between a single cigarette smoke-derived substance, induction of a novel chemoattractant, and smoking-related interstitial lung diseases. An increase in pulmonary OPN could result in enhanced survival and increased influx of macrophages and dendritic cells. Because the accumulation of macrophages and Langerhans cells is one hallmark of smoking-related interstitial lung diseases, high OPN-production may be a key factor for induction and progression of these diseases.

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