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Lung

Oxidized Phosphatidylcholine in Alveolar Macrophages in Idiopathic Interstitial Pneumonias

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Abstract. It has been suggested that oxidative stress plays a pathogenic role in idiopathic interstitial pneumonias. Macrophage- or neutrophil-derived oxidants seem to be important sources of oxidative stress in this group of inflammatory disorders. Recent experimental studies have revealed that oxidative injury during inflammation or apoptosis can change phosphatidylcholine of cell membrane into its oxidized form, which serves as a ligand for macrophage scavenger receptor CD36. Recently, we developed a monoclonal antibody against oxidized phosphatidylcholine. Using this novel antibody, we performed an immunohistochemical investigation to clarify the localization of oxidized phosphatidylcholine in lung tissues of idiopathic interstitial pneumonias and a relationship between oxidized phosphatidylcholine localization and CD36 expression. Lung specimens obtained from patients with desquamative ($n = 8$) or usual interstitial pneumonia ($n = 15$) were studied. Thirteen normal lung tissues were also examined as controls. Antibodies against oxidized phosphatidylcholine, CD36, epithelial cells, macrophages, and neutrophils were used as primary antibodies. The positive cell number was counted by computer-aided morphometry. While there were no oxidized phosphatidylcholine-positive cells in normal lungs, lungs of desquamative or usual interstitial pneumonia contained large numbers of oxidized

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phosphatidylcholine-positive cells in the alveolar spaces. Double-staining analysis revealed that most oxidized phosphatidylcholine-positive cells were macrophages. The oxidized phosphatidylcholine-positive cells were increased in association with the increase in the densities of macrophages (Rs = 0.87, $p < 0.0001$) and neutrophils (Rs = 0.89, $p < 0.0001$). Accumulated macrophages also showed distinct CD36 expression. These findings suggest that oxidative stress and the related product, oxidized phosphatidylcholine, play an important role in the pathophysiology of idiopathic interstitial pneumonias.

Key words: Oxidized phosphatidylcholine—Scavenger receptor—Macrophages—Oxidative stress—Idiopathic interstitial pneumonias.

Introduction

Idiopathic interstitial pneumonias (IIPs) are a group of interstitial lung diseases of unknown etiology, characterized by parenchymal cell injury and fibrosis of the alveolar septa with accumulation of alveolar macrophages and neutrophils in the distal airspaces [1]. Idiopathic pulmonary fibrosis/usual interstitial pneumonia (UIP) and desquamative interstitial pneumonia (DIP) are two major entities of IIPs. DIP affects primarily cigarette smokers and generally has a good prognosis. The diseased lung in DIP shows increased numbers of macrophages evenly dispersed within alveolar spaces. In contrast, the prognosis of UIP is extremely poor. The histological features of UIP are architectural destruction, fibrosis often with honeycombing, scattered fibroblastic foci, patchy distribution and involvement of the periphery of the acinus or lobule. Despite the differences in the clinical and histological features between DIP and UIP, oxidative stress, caused by impaired oxidative/antioxidative balance, has been suggested to be a common pathogenic factor in both types of IIPs [2,3].

Inhaled exogenous radicals, including reactive oxygen species (ROS), cigarette smoke and nitrogen dioxide, are recognized to be important sources of oxidative stress in lung [4]. In addition, accumulated macrophages and neutrophils seem to participate in the oxidative stress mechanism as endogenous sources [4,5]. These inflammatory cells release various mediators that induce pulmonary injury and fibrosis, such as cytokines, growth factors and proteases [6, 7]. Macrophages and neutrophils also generate ROS and free radicals, which can provoke oxidative lung injury [5,8]. Myeloperoxidase (MPO), for example, a typical oxidant-producing enzyme secreted by neutrophils [9], has been reported to be present at a higher concentration in bronchoalveolar lavage fluid from IIP patients than in that from normal subjects [10].

Lipid peroxidation can occur in the course of oxidative cell/tissue injury, and is considered a useful indicator of oxidative stress status in various lung diseases [11–13]. Oxidized phosphatidylcholine (oxPC), one of such lipid peroxides, is generated by oxidative modification of phosphatidylcholine (PC). Because PC is an essential component of plasma membranes of every cell type, it is easy to

speculate that oxPC may be detectable in oxidatively damaged cells. In fact, oxPC generation has been found in association with apoptosis induced by oxidation [14–16]. Importantly, recent experimental studies have suggested a role of oxPC generation in inflammatory/immune responses; oxPC may trigger damaged cell clearance by macrophages via binding to CD36, a member of macrophage class B scavenger receptors [16–18]. In addition, PC is contained in alveolar fluid as an indispensable part of surfactant phospholipids, and previous studies have revealed differences in the surfactant phospholipid profile between normal and diseased lungs [19]. In IIP lungs, unsaturated forms of PC, which are thought to be more easily oxidized than the saturated form, are increased [19]. Oxidation of surfactant PC may occur during the inflammatory process and may contribute to the pathological mechanism of IIPs.

These experimental findings and clinical observations collectively evoke a special interest in oxPC localization in IIPs. However, to the best of our knowledge, there has been no report concerning oxPC localization in normal and diseased human lung tissues. Recently, we developed a novel anti-oxPC antibody [20], and have demonstrated the localization of oxPC in macrophages in human atherosclerotic lesions by using this antibody [21]. In this study, we immunohistochemically investigated the localization of oxPC and the expression of CD36 in human lung tissues with DIP or UIP.

Materials and Methods

Lung Tissue Samples

Surgical (thoracoscopic or open) lung biopsy specimens obtained from patients with DIP ($n = 8$) or UIP ($n = 15$) were studied (Table 1). The diagnoses of DIP and UIP were made according to the latest criteria [1]; the patients who were untreated at the time of the biopsy presented the clinical and radiological features of DIP or UIP, including findings in high-resolution computed tomography, and their biopsy specimens showed the DIP or UIP pattern histologically. All 8 DIP patients were cigarette smokers, and Brinkman indices were greater in DIP patients than in UIP patients ($p = 0.06$). Most UIP patients showed a characteristic restrictive pattern of ventilatory defect with impaired gas exchange. These pulmonary function abnormalities were mild in DIP patients. Normal lung tissues from 13 autopsied patients (all non-smokers) without any pulmonary disorders were examined as controls.

These tissue blocks were fixed in formalin and embedded in paraffin. Thirty serial sections were cut from each block at 4-μm thickness. Every first two sections were stained with hematoxylin-eosin (HE) for morphological examination, and Azan-Mallory for evaluation of collagen deposition. The other sections were used for immunohistochemical staining analysis.

In addition, frozen lung sections were made because the monoclonal antibody against CD36 did not work on formalin-fixed sections, but worked well on frozen sections. The frozen tissues were available from 3 of the 13 normal lung specimens, one of the 8 DIP samples, and 3 of the 15 UIP samples were sectioned serially at 7-μm thickness and fixed in acetone.

The Ethical Committee of Osaka City University Hospital approved this study and informed consent was obtained from every patient or family.

Immunostaining

To identify oxPC, a newly developed mouse monoclonal antibody, DLH3, was used. The methods of antibody production and specificity testing have been reported previously [20]. Macrophages,

Table 1. Clinical characteristics of the patients studied

Patient no	Sex (F/M)	Age (yrs)	Brinkman index	%VC (%)	%DLco (%)	PaO2 (Torr)
UIP						
1	F	70	0	64.7	51.5	88.3
2	M	49	600	85.2	77.9	89.4
3	M	47	1200	96.2	54.0	86.8
4	M	47	0	37.7	34.2	71.8
5	F	71	0	73	34.1	71.8
6	F	57	0	71.7	44.7	75.7
7	M	61	600	64.7	34.5	63
8	F	63	0	95.1	40.6	80.7
9	M	54	600	46.7	33.3	67.9
10	M	53	720	79	35.7	75.8
11	M	50	1500	51	46	75.8
12	M	67	400	73.2	43.9	81.5
13	F	53	300	76	57	74.3
14	M	68	480	61.5	39.2	56
15	M	65	1200	71	47.3	76
Mean ± SD	5/10	58 ± 9	507 ± 490	69.8 ± 16.4	44.9 ± 11.9	75.7 ± 9.1
DIP						
16	M	60	830	92	51.7	94.6
17	M	50	900	109	52.6	100.6
18	M	46	780	118	54.9	86.7
19	M	29	540	27.9	18.5	63.3
20	M	67	1400	99	66.4	73.7
21	M	48	1160	95.5	66.6	96.3
22	M	57	680	79	35	69.4
23	M	59	500	53.9	48.7	68
Mean ± SD	0/8	52 ± 12	849 ± 306	84.3 ± 30.0	49.3 ± 16.1	81.6 ± 14.7
Control						
Mean ± SD	4/6	58 ± 18	0	ND	ND	ND

UIP: usual interstitial pneumonia; DIP: desquamative interstitial pneumonia; VC: vital capacity; DLco: carbon monoxide diffusion capacity of the lung; Pao2: arterial oxygen tension; F: female; M: male; ND: not done.

neutrophils, red blood cells (RBCs), epithelial cells and apoptotic cells were identified with the specific antibodies listed in Table 2. For the identification of CD36, frozen sections were used with anti-CD36 antibody.

Single Staining. Sections were incubated with one primary antibody, either overnight at 4°C or for 1 hour at room temperature. The sections were then subjected to a 3-step staining procedure using streptavidin-biotin complex with horseradish peroxidase for color detection. Horseradish peroxidase activity was visualized with 3-amino-9-ethylcarbazole. Finally, sections were faintly counterstained with hematoxylin. The specificity and results obtained with DLH3 were checked by omission of the primary antibody and use of a non-immune mouse IgG antibody (Dako Cytomation A/S, Denmark) as a negative control. Human atherosclerotic plaque tissues with macrophage-derived foam cells served as a positive control [21].

Double Immunostaining. For the identification of cell types that showed oxPC positivity, we performed double immunostainings for oxPC/macrophage (PG-M1), oxPC/neutrophil (MPO), and oxPC/epithelial cell (cytokeratin). In these double immunostainings, alkaline phosphatase was

Table 2. Primary antibodies used in this study

Designation	Clone or catalog number	Cell identified	Source	Tissue fixation	Working dilution
oxPC	DLH3	-	Itabe et al. [20]	Formalin	1/50
CD68	PG-M1	Macrophages	Dako	Formalin	1/50
HAM56	HAM56	Macrophages	Dako	Formalin	1/30
CD66	Kat4c	Neutrophils	Dako	Formalin	1/200
MPO	A0398	Neutrophils	Dako	Formalin	1/200
Neutrophil elastase (NE)	NP57	Neutrophils	Dako	Formalin	1/100
Glycophorin A	JC159	RBCs	Dako	Formalin	1/100
Surfactant apoprotein-A (SP-A)	PE10	Pneumocytes	Dako	Formalin	1/100
Cytokeratin	AE1 + AE3	Epithelial cells	Dako	Formalin	1/50
Single stranded DNA (ssDNA)	A4506	Apoptotic cells	Dako	Formalin	1/100
CD68	EBM11	Macrophages	Dako	Frozen	1/200
CD36	Fab-152	-	Immunotech	Frozen	1/2000

Dako: Dako Cytomation A/S, Denmark; Immunotech: Immunotech, Marseille, France.

visualized with fast blue BB (blue; PG-M1, MPO, and cytokeratin) and peroxidase was visualized with 3-amino-9-ethylcabazole (red; oxPC). To analyze oxPC localization in apoptotic cells, double immunostaining for oxPC (red)/single stranded DNA (ssDNA; blue) was also performed.

Morphometry

The density of oxPC-positive cells, macrophages or neutrophils in the alveolar spaces was quantified by computer-aided morphometry [21] and expressed as cell counts per 1 mm^2 alveolar area. Intraobserver variability was determined from triplicate measurements. The mean ± SD difference among measurements was 3.6 ± 0.7%. The data were statistically analyzed with the Mann-Whitney U-test or Spearman's rank correlation coefficient (Rs). P values of < 0.05 were considered significant.

Results

Normal Lungs

In normal lung tissues, no significant inflammatory changes were found (Fig. 1A), and only a few macrophages were detected in the alveolar spaces (Fig. 1B). There were no oxPC-positive cells in the normal lungs (Fig. 1C). There were no neutrophils infiltrated in the alveolar spaces (Fig. 1D). Frozen sections showed CD36 immunoreactivity in endothelial cells of microvessels, but not in alveolar macrophages (Fig. 2).

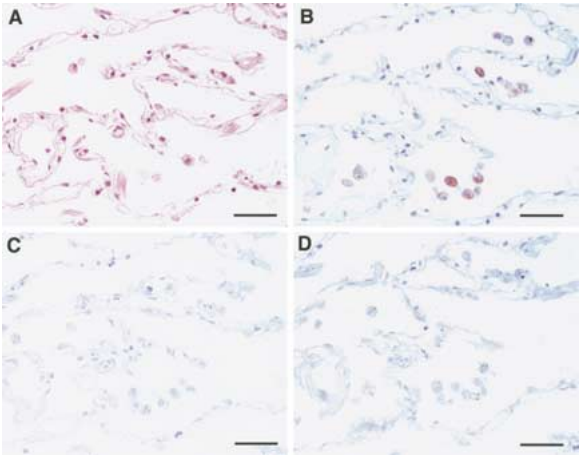

Fig. 1 Histological and immunohistochemical sections of normal lung tissue. (**A**) HE section shows thin alveolar walls without significant inflammatory changes. (**B**) Immunostaining for macrophages (PG-M1, red). There are a few macrophages in the alveolar spaces. (**C**) An adjacent section stained for oxPC (DLH3, red). No oxPC-positive cells are found in the alveolar spaces. (**D**) An additional adjacent section stained for neutrophils (MPO, red). Neutrophils are not found in the alveolar spaces, but a few are seen in capillary lumens. Scale bars indicate 50 μm.

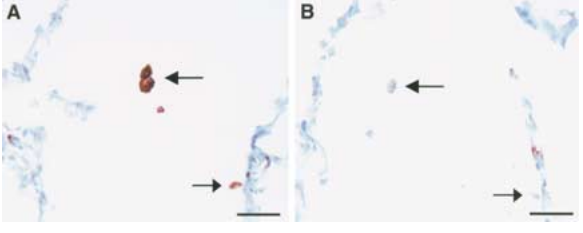

Fig. 2 Immunohistochemical frozen sections of normal lung tissue. (**A**) A frozen section stained for macrophages (EBM11, red; arrows). (**B**) An adjacent frozen section stained for CD36 (red). Only microvascular endothelial cells are stained. Macrophages are negative for CD36 (arrows). Scale bars indicate 50 μm.

DIP Lung

DIP lungs showed diffuse and dense inflammatory cell accumulation in the alveolar spaces (Fig. 3A), and immunohistochemical examination revealed that macrophages were the major cell type accumulated in the alveolar spaces (Fig. 3B). There were many oxPC-positive cells in the alveolar spaces filled with abundant macrophages (Fig. 3C). Double immunostainings for oxPC/macrophages, oxPC/neutrophils, and oxPC/epithelial cells revealed that most oxPC-positive cells were macrophages (Fig. 3D). Neutrophils (MPO, NE and CD66-positive cells) were also present in the intra-alveolar cell clusters (not shown). Exfoliated epithelial cells

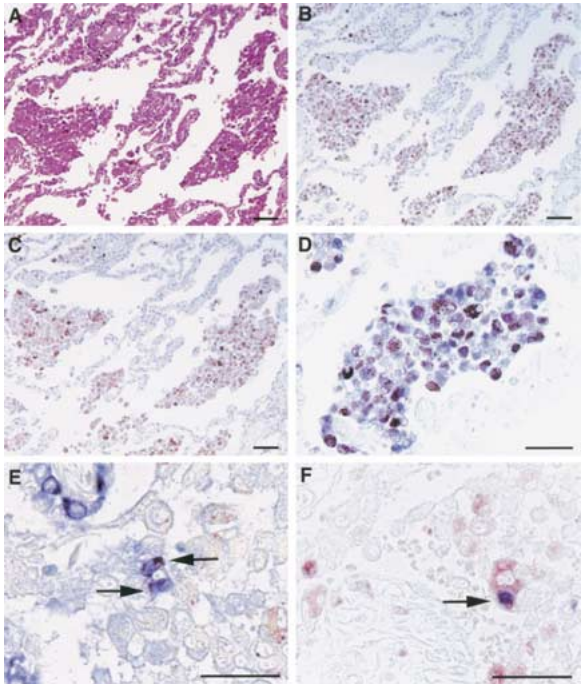

Fig. 3 Histological and immunohistochemical sections of DIP lung. (**A**) HE section shows accumulation of abundant inflammatory cells in the alveolar spaces. (**B**) An adjacent section stained for macrophages (PG-M1, red) shows marked accumulation of alveolar macrophages. (**C**) An additional adjacent section stained for oxPC (red). Abundant oxPC-positive cells are observed in the alveolar spaces. (**D**) Double immunostaining for macrophages (blue) and oxPC (red). Most cells show double staining (purple), indicating oxPC localization in alveolar macrophages. (**E**) Double immunostaining for epithelial cells (cytokeratin, blue) and oxPC (red). Some exfoliated epithelial cells are positive for oxPC (purple; arrows). (**F**) Double immunostaining for ssDNA (blue) and oxPC (red). An oxPC-positive cell has a nucleus with immunoreactivity for ssDNA (arrow). Scale bars: A, B and C = 100 μm. D, E and F = 50 μm.

(SP-A and cytokeratin-positive) were occasionally present in the airspaces, and some of these epithelial cells were positive for oxPC (Fig. 3E). A few oxPC-positive cells had nuclei with immunoreactivity for ssDNA (Fig 3F), indicating that oxPC-positivity was, in part, related to apoptosis of damaged cells. Extravasation of RBCs, which was confirmed by immunostaining for glycophorin A, was seen near the clusters of oxPC-positive macrophages (not shown).

A frozen sample obtained from one of the 8 DIP lungs was investigated with immunohistochemistry for CD36. Distinct positivity for CD36 was observed in accumulated macrophages in the alveolar spaces (Fig. 4).

UIP Lungs

UIP lungs showed heterogeneous distribution of interstitial fibrosis and inflammation (Fig. 5A), and some of them contained areas of honeycomb change.

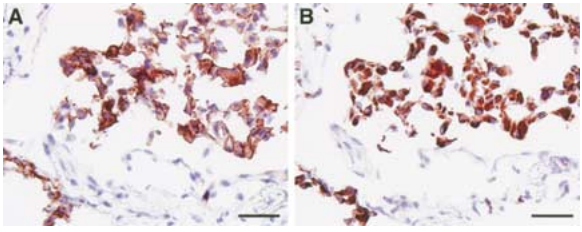

Fig. 4 Immunohistochemical frozen sections of DIP lung tissue. (**A**) A frozen section stained for macrophages (EBM11, red). Many macrophages are accumulated in the airspaces. (**B**) An adjacent frozen section stained for CD36 (red). Accumulated macrophages are mostly positive for CD36. Scale bars indicate 50 μm.

Inflammatory cell accumulation in airspaces was also observed, but it was milder than in DIP lungs. Immunohistochemical examination revealed that the cells accumulated in the airspaces were predominantly macrophages (Fig. 5B). Moreover, there were many oxPC-positive cells in the airspaces (Fig. 5C), and double immunostaining analysis revealed that most of the oxPC-positive cells were macrophages (Fig. 5D).

Frozen samples obtained from three of the 15 UIP patients were subjected to CD36 immunohistochemistry. These contained CD36-positive alveolar macrophages (Fig 6). However, the CD36-positive macrophages were fewer in the UIP lungs than those in the DIP lung.

Morphometry

As shown in Figure 7, the densities of oxPC-positive cells, macrophages and neutrophils in the alveolar spaces were significantly greater in both DIP (oxPC-positive cells, $p < 0.0001$; macrophages, $p < 0.001$; neutrophils, $p < 0.001$) and UIP (oxPC-positive cells, $p < 0.0001$; macrophages, $p < 0.0001$; neutrophils, $p < 0.0001$) lungs than in normal lungs. Moreover, the densities of these intra-alveolar cells were significantly greater in DIP lungs than in UIP lungs (oxPC-positive cells, $p < 0.001$; macrophages, $p < 0.001$; neutrophils, $p < 0.005$). The difference in smoking history did not affect these morphometric data in UIP cases. The density of oxPC-positive cells was correlated with the density of macrophages ($Rs = 0.87$, $p < 0.0001$) and the density of neutrophils ($Rs = 0.89$, $p < 0.0001$).

Discussion

Oxidative injury has been considered to be implicated in the pathological mechanism of IIPs [2, 3, 5, 11]. Cigarette smoking is known to be one of the most common causes of oxidative lung injury [4, 22]. In our series of DIP or UIP, most patients were smokers, as described in the previous reports [1]. Within the lungs, alveolar macrophages and neutrophils are considered to be the important cell

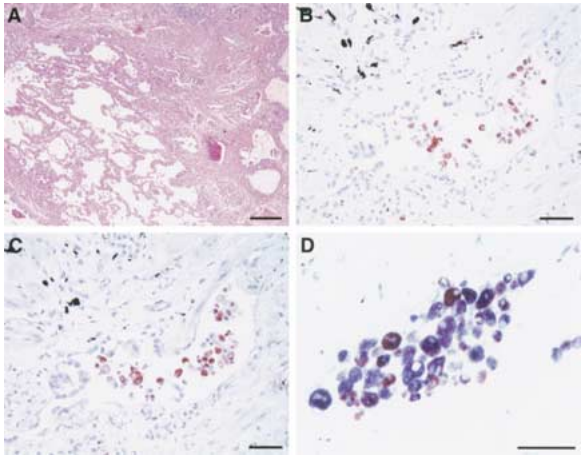

Fig. 5 Histological and immunohistochemical sections of UIP lung. (**A**) HE section at low magnification shows a heterogeneous distribution of interstitial fibrosis and inflammation. (**B**) An adjacent section stained for macrophages (PG-M1, red) shows macrophage accumulation in the airspace. (**C**) An additional adjacent section stained for oxPC (red). OxPC-positivity is seen in the cell cluster. (**D**) Double immunostaining for macrophages (blue) and oxPC (red). Macrophages in the alveolar space are positive for oxPC (purple). Scale bars: A = 250 μm; B, C and D = 50 μm.

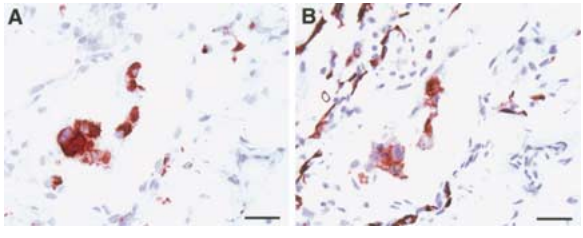

Fig. 6 Immunohistochemical frozen sections of UIP lung tissue. (**A**) A frozen section stained for macrophages (EBM11, red). Macrophage accumulation is observed in the airspaces. (**B**) An adjacent section stained for CD36 (red). Similar to DIP lung, accumulated macrophages show distinct positivity for CD36. Scale bars indicate 50 μm.

types in endogenous oxidant generation [4]. MPO released from neutrophils serves as a potent enzymatic catalyst of lipid peroxidation at sites of inflammation and augments oxidative stress [9]. Our immunohistochemical study revealed a dense accumulation of macrophages and neutrophils in the alveolar spaces of IIP lungs. The accumulated neutrophils were positive for MPO throughout. Thus, oxidative potency was presumably augmented in both DIP and UIP lungs.

The intense oxidation potency in the IIP lungs was also indicated by the presence of abundant oxPC-positive cells. This is the first study to demonstrate the localization of oxPC in human lung tissues and a close relationship between oxPC localization and IIPs. Because oxPC is a lipid peroxide indicating oxidative cell/tissue injury [14,16], these findings suggest that oxidative stress actually

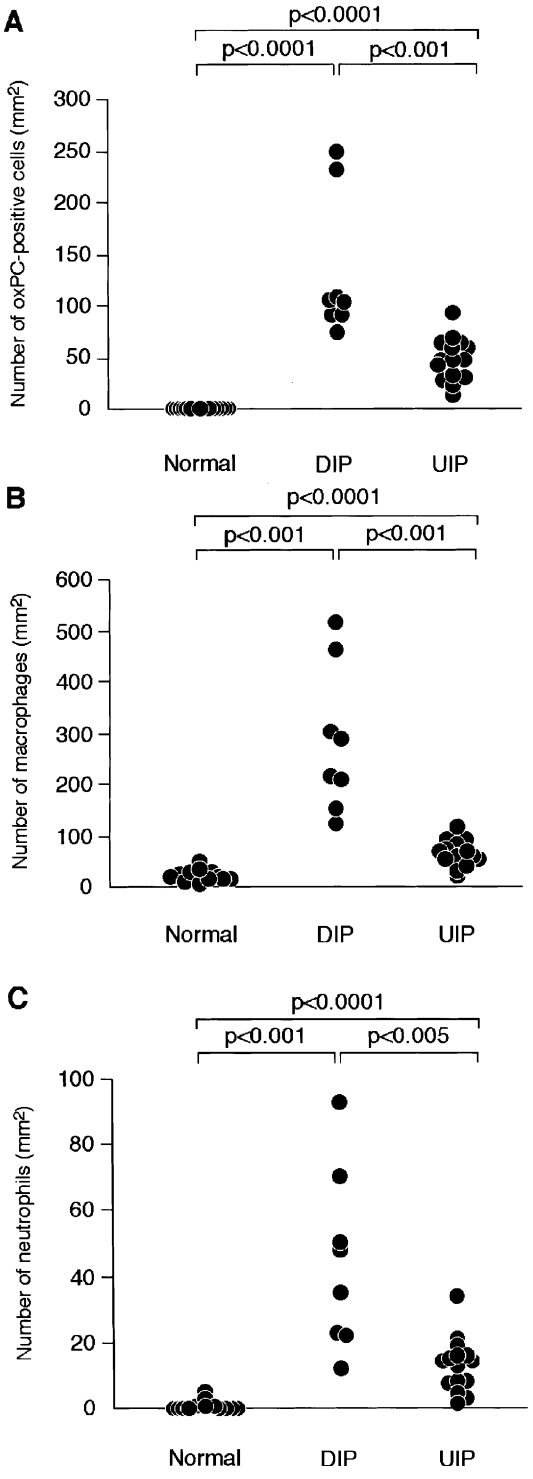

Fig. 7 Results of morphometric analyses in UIP, DIP and normal lungs. (**A**) The density of oxPC-positive cells in the alveolar spaces is significantly increased in DIP and UIP lungs compared with that in normal lungs. (**B**) The density of alveolar macrophages is significantly higher in DIP and UIP than in normal lungs. (**C**) The density of neutrophils in the alveolar spaces is significantly higher in DIP and UIP than in normal lungs.

participates in the pathological mechanism of IIPs. In addition, the amount of oxPC-positive cells increased in association with the increase in the densities of macrophages and neutrophils accumulated in the alveolar spaces suggests that macrophages and neutrophils contribute to the process of oxidative stress leading to oxPC generation in IIP lungs.

Our double immunostaining technique revealed that most of the oxPC-positive cells were macrophages. Recent evolution in the research field of these oxidized phospholipids, mainly by Steinberg et al. suggests that oxPC may be a key factor in phagocytotic uptake by macrophages [15–18, 23–26]. Therefore, oxPC present in alveolar macrophages is likely to have been internalized as phagocytosis rather than generated in the macrophages *in situ*. Many types of cell surface receptors have been found on macrophage, and some of them recognize oxPC as a ligand [16]. CD36 is a member of macrophage class B scavenger receptors, and participates in the phagocytotic action of macrophages [26]. Accumulating evidence has raised a concept that oxPC serves as a ligand for CD36, and the oxPC-CD36 binding plays a key role in the clearance of futile and harmful metabolites or senescent and dysfunctional cells that contain oxPC [15–18, 23–26]. We found that increased numbers of macrophages in the alveolar spaces expressed CD36 in DIP and UIP lungs, suggesting that the CD36-mediated scavenging activity of macrophages appears to be strengthened in association with oxPC generation in DIP and UIP lungs. In addition, our observations that DIP lungs contained more CD36- and oxPC-positive macrophages than UIP lungs may reflect a difference in the pathological mechanism between DIP and UIP. However, further study is necessary to clarify the pathological significance of oxPC localization in DIP versus UIP.

The remaining question is the origin of oxPC in IIP lungs. As described above, PC is a substantial element of alveolar surfactant and thus it is possible that oxPC is generated from surfactant by oxidative modification related to inflammatory reactions. Alveolar surfactant contains unsaturated phospholipids, which can be oxidized by experimental ozone exposure [27]. Because unsaturated PC content in alveolar surfactant is increased in IIP lungs [19], oxPC might be easily generated from surfactant PC. In animal models of bleomycin-induced pulmonary fibrosis, phospholipids, including PCs, have been noted to be increased in alveolar surfactant and alveolar macrophages [28, 29]. The results of these previous studies can partially explain our finding in IIP lungs' accumulation of alveolar macrophages containing oxPC. As the second possibility, we should consider that oxPC originated from injured cells. As described above, recent *in vitro* studies have reported that oxPC is generated on plasma membranes of cells suffering from oxidative or inflammatory damage [14–16]. Our immunohistochemical double-staining analysis revealed occasional but distinct oxPC localization in apoptotic cells that supports the previous *in vitro* findings. Roughly, there were two types of nucleated cells in the airspaces: clustered inflammatory cells in DIP and UIP, and a few exfoliated epithelial cells in DIP. It is well known that neutrophil degeneration and apoptosis occur subsequently to various inflammatory disorders [30]. In the present study, we found marked neutrophil infiltration in the alveolar spaces in both DIP and UIP and a sig-

nificant correlation between the number of neutrophils and oxPC-positive cells in the alveolar spaces. Infiltrated neutrophils may not only be a cause of oxidative injury but also a provider of oxPC. Alternatively, we found a few exfoliated epithelial cells in the airspaces of DIP lungs and some of these cells showed immunoreactivity for oxPC. These findings suggest that damaged and exfoliated epithelial cells can also be a provider of oxPC. In addition, we found that RBCs leaked into the alveolar spaces and were surrounded by oxPC-positive macrophages, suggesting that plasma membranes of hemorrhaged and degenerated RBCs were also one of the sources of oxPC. This interpretation can be supported by a previous experimental study concerning the phagocytotic pathway of damaged RBCs [23]. Collectively, it is presumed that oxPC contained in accumulated macrophages in IIP lungs may be originated under the inflammatory condition from alveolar surfactant exposed oxidative stress and from plasma membranes of oxidatively damaged cells.

In conclusion, this study demonstrates prominent oxPC localization in the alveolar spaces and a close relationship between oxPC localization and inflammatory cell accumulation in DIP and UIP lungs. Enhanced oxPC localization was linked to up-regulation of CD36 expression in alveolar macrophages, suggesting that oxidative injury plays an important role in the inflammatory process, and the oxidation product, oxPC and its receptor, CD36, may contribute to the pathological mechanism of IIPs.

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