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ORIGINAL ARTICLE

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Immunohistological detection of the β subunit of prolyl 4-hydroxylase in rat and mini pig lungs with radiation-induced pulmonary fibrosis

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Abstract Polyclonal and monoclonal antibodies to the β subunit of prolyl 4-hydroxylase, the protein disulphide isomerase, were used to compare the pulmonary cells in 13 normal and in 20 fibrotic rat and mini-pig lungs made fibrotic by X-ray irradiation, using the ABC immunoperoxidase technique. In normal lungs, prominent staining of Clara cells and type II pneumocytes and weaker reactivity with alveolar macrophages, fibroblasts, endothelial and smooth muscle cells were detectable. In pulmonary disease, in which interstitial fibrosis was the characteristic feature, the immunoreactivity was increased in both the epithelial and interstitial cells. Type I pneumocytes remained negative. In the early stages of disease (3 to 4 weeks after irradiation) when little morphological alteration was seen, capillary endothelial cells had already become immunoreactive. These results underline the complex involvement and interaction of different lung cell populations in the process of pulmonary fibrogenesis.

Key words Prolyl 4-hydroxylase · Pulmonary fibrosis
Alveolar epithelium · Protein disulphide isomerase

Introduction

The formation of collagen fibrils depends on the hydroxylation of proline occurring in newly synthesized procollagen molecules. The β subunit of the enzyme prolyl 4-hydroxylase, the protein disulphide isomerase (PDI; EC 1.14.11.2) catalyses this essential step (for review see [12]). Because altered collagen metabolism is known to play a central role in pulmonary fibrosis [1], we were interested in the localization of the PDI in lung tissues under normal and pathological conditions. In a recent study, we reported on the selective immunohistological detection of PDI in human type II alveolar cells and bronchial epithelial cells after microwave treatment of routinely formalin-fixed, paraffin-embedded tissue [16].

Our aim in this study was to evaluate rat and mini pig models of radiation-induced fibrosis for the presence of the PDI and to clarify the involvement of epithelial cells in this kind of lung injury.

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Materials and methods

The right lungs of adult Fischer rats weighing 250–300 g were irradiated with a single dose of 16 Gy ultrahard X-ray (for details see [13, 14]). The animals were sacrificed at 1, 3, 6, 24, 48 h, 7, 14, 28 days, 2, 3 and 6 months after irradiation. As control, untreated animals (n=11) of the same age were used.

Lungs of adult mini pigs weighing 90–100 kg were treated on five consecutive days with 5×6, 5×7, 5×8 Gy. The animals (n=9) were sacrificed 9 months after irradiation (for details see [15]). Two control animals were included.

The mini-pig lung samples were fixed with 4% neutral buffered formaldehyde. Rat lungs were cannulated through the trachea and fixed with Schaffer's solution under 20 cm water pressure. After embedding in paraffin, the 5 μm-thick sections were cut and processed for immunohistochemistry.

The mouse monoclonal antibody 1D3 was raised against a peptide corresponding to the carboxyterminal 12 residues of rat PDI (KDDDQKAVKDEL). The specificity of the 1D3 antibody was determined by immunoblotting as described in Huovila et al. [10]. Rat liver fractions were prepared as described in Sztul et al. [29] and blotted with 1D3 supernatant for 2 h before visualization by alkaline phosphatase coupled goat antibody (Medac, Hamburg, Germany). Comparison of the total microsomal fraction (Ms) with a Golgi fraction depleted of endoplasmic reticulum (ER) (Go) and an ER enriched fraction (ER) reveals that the single reactive band is localized to the ER (Fig. 1). This localization and the molecular weight of the band show that 1D3 is a specific marker of PDI in rat tissue.

For further confirmation of the PDI immunostaining in rats a polyclonal antiserum against the PDI was included. This rabbit antiserum was raised against bovine PDI purified by the method of Lambert and Freedman [21] as described previously [30]. In contrast to the monoclonal antibody 1D3, no cross-reactivity with mini pig tissue was found.

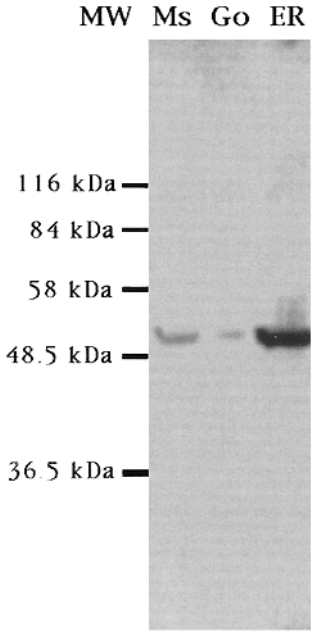

Fig. 1 Immunoblotting of rat liver fractions with 1D3. The specificity of the antibody is seen by the comparison of total microsomes (*Ms*), Golgi enriched fractions (*Go*) and endoplasmic reticulum enriched fractions (*ER*). The positions of migration of the prestained molecular weight markers (Sigma Cat# SDS 7B) are indicated (*MW*)

Table 1 PDI immunoreactivity of rat lung cells at different time after irradiation (16 Gy) (+, weak; ++, moderate; +++, strong; ++++ very strong immunoreactivity)

	Type II cells	Capillary endothelia	Fibroblasts	Alveolar macrophages
2d	++	++	++	++
7d–21d	++	+++	++–+++	
28d	+++	++–+++	+++	++–+++[b]
2 months	++++[a]	+++	+++	+++[b]
3–12 months	++++[a]	+++	+++	++[b]
Control	++	+	+	++

[a] Hyperplastic cells
[b] Increased number of cells including cluster formation

For immunohistochemistry paraffin sections were mounted on slides that were coated with silan. After drying overnight, the sections were dewaxed, and irradiated with microwave in 0.01 M sodium citrate buffer (pH 6.0), 2×5 min at 750 W.

The mouse monoclonal antibody against PDI (1D3) was detected with a commercially available ABC technique (Vectastain Elite kit, Vector Laboratories, Burlingame, Calif., USA) according to the protocol of the producer. The dilution of the hybridoma supernatant was 1:10 after microwave treatment (without microwave or protease pretreatment a weak but specific staining signal was found; Kasper, unpublished data). At this concentration, the antibody is specific as shown by peptide competition, immunofluorescence and immunoblotting. Negative controls consisted of omission of the primary antibody and substitution by irrelevant monoclonal antibodies of similar isotypes. For the detection of the polyclonal antibody, an indirect immunoperoxidase technique was used. After washing in phosphate buffered saline (PBS), pH 7.4, for 5 min, the sections of rat lungs were treated with 0.3% hydrogen peroxide for 30 min, incubated with 30% fetal calf serum and then incubated with the rabbit polyclonal antiserum, dilution 1:20. The antibodies were detected by the use of peroxidase-coupled goat anti-rabbit IgG (HRP 77; Dr. H. Großmann, dilution 1:400). The peroxidase activity was visualized with diaminobenzidine. Negative controls included omission of the primary antibody and its replacement by PBS or normal rabbit serum.

For double label immunofluorescence microscopy, sections of normal rat lung were rinsed in PBS and incubated with the 1D3 antibody followed by DTAF-coupled goat anti-mouse Ig (Dianova, Hamburg, Germany), dilution 1:80. Then followed a second staining using the polyclonal rabbit anti cytokeratin antiserum 1146, dilution 1:160 (kindly provided by Dr. G.N.P. Van Muijen, Department of Pathology, University of Nijmegen, Nijmegen, The Netherlands) or a polyclonal rabbit anti surfactant protein A (SP-A) antiserum, dilution 1:80 (kind gift from Dr. K. Sakai, Department of Nutrition, University of Tokushima, Tokushima, Japan) and Texas Red-coupled goat anti rabbit IgG (Dianova), dilution 1:40. In addition, the biotinylated lectins *Dolichos biflorus* agglutinin (DBA; a marker of rat alveolar macrophages, [13]), *Bauhinia purpurea* lectin (BPA; type I pneumocyte- and macrophage- specific, [17]) were used followed by Texas Red-labelled avidin, 1:200 diluted (Vector Laboratories) or *Maclura pomifera* agglutinin (MPA), fluorescein isothiocyanate-labelled (Medac; dilution 1:200). In control experiments, the binding of the lectins was inhibited after preincubation of the lectin with a 0.2 M solution of the corresponding inhibitory sugar.

In paraffin sections of mini pig lungs, the immunoreactivity of the antibody 1D3 was additionally tested employing the indirect immunofluorescence technique (see above).

Results

The distribution of the β subunit of prolyl 4-hydroxylase was studied by indirect immunoperoxidase (rats only) or ABC technique in the lung tissue from all of the 33 rats and mini pigs (Tables 1, 2). There were no qualitative

Table 2 Dose dependent immunoreactivity of mini pig lung cells with PDI-specific monoclonal antibody 1D3

[a] Cuboidal metaplasia
[b] Marked hyperplasia of type II pneumocytes;
[c] In addition, flattened epithelial (type II?) cells with strong staining pattern indicating incomplete differentiation to type I cells (compare (Fig. 4d)

	Experimental group			Control
	5×6 Gy	5×7 Gy	5×8 Gy	
1. Capillary endothelia staining	+	+–++	++	+
2. Alveolar macrophage staining	+–++	++	+++	++
3. Fibroblast staining	+–++	++	++–+++	+
4. Type II cell staining	+++	++++[a, c]	++++[a, b, c]	+++

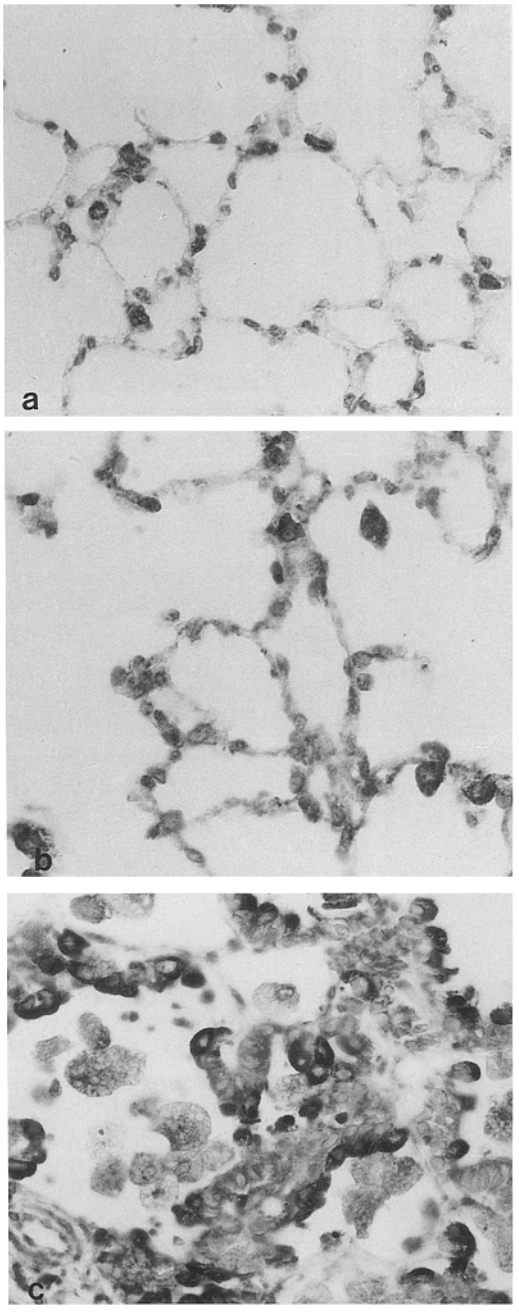

Fig. 2a Normal rat lung. Paraffin section. Prominent staining of type II pneumocytes and weak staining of capillary endothelial cells by using monoclonal antibody 1D3 against PDI. **b** Irradiated rat lung (4 weeks after X-ray application) with enhanced immunoreactivity for PDI in endothelial cells and type II pneumocytes. **c** Irradiated rat lung (6 months after irradiation) with strong PDI staining of proliferative, hyperplastic type II pneumocytes, of interstitial cells and of alveolar macrophages. ABC technique with weak Mayer's haematoxylin counterstain. **a–c** ×175

differences between the polyclonal antiserum and the monoclonal antibody 1D3 in immunostaining of rat lung tissues (not shown).

In the normal rat tissues, there was evidence of PDI immunoreactivity in type II pneumocytes, in alveolar macrophages and to a lesser extent in endothelial cells and in smooth muscle cells (Figs. 2a; 3). In addition, the bronchiolar Clara cells were apically PDI-positive as revealed by double immunofluorescence for PDI and surfactant protein A (Fig. 3g, i). The focal staining of Clara cells for SP-A (Fig. 3h) is not an artefact. The remaining unstained Clara cells were positive for SP-D as seen in SP-A/SP-D double staining (not shown; Kasper, unpublished data). However, the ciliated bronchial epithelial cells found in larger bronchi were devoid of immunoreactivity for PDI (not shown). Furthermore, the alveolar brush cells and the type I pneumocytes failed to stain with the PDI-specific antibodies (Fig. 3a–d). A subpopulation of alveolar macrophages with binding pattern for the lectin DBA exhibited a very weak PDI immunoreactivity (Fig. 3i–j) when compared with the strong reaction of the MPA binding alveolar macrophages (Fig. 3e–f). In addition, staining for PDI was found in mesothelial cells (Fig. 3e). A similar staining pattern of pulmonary cells could be detected in normal mini pigs (Figs. 4a, 5a), employing the monoclonal antibody 1D3 only. The type II cells, however, showed stronger immunoreactivity compared with the normal rat cells.

In the present study, interstitial pulmonary fibrosis was a characteristic histological feature of irradiated animals (Table 1, 2). In rat lung specimens obtained early in the disease course (at about 3 to 4 weeks after irradiation) there was increased immunoreactivity of the capillary endothelial cells in the slightly expanded interstitium (Fig. 2b). Immunoreactive alveolar macrophages also were increased and enlarged in the widened interstitium and alveolar septa (Fig. 2b). Lung specimens obtained late in the course of the disease (3 months to 12 months) revealed a dense accumulation of PDI in both the interstitial as well as the epithelial cells, except in the type I pneumocytes. The lung samples showed strong deposits of the enzyme in the hyperplastic and proliferative alveolar epithelium (Table 1), to a lesser extent in alveolar macrophages (Fig. 2c) and in the interstitium (not shown). In mini pigs, a dose dependent increase of PDI has been found (Table 2). The enhanced immunoreactivity comprised cuboidal and hyperplastic type II pneumocytes, Clara cells, alveolar macrophages, myofibroblasts, smooth muscle and endothelial cells (Fig. 4b–d, Fig. 5). In contrast, type I cells or their remnants were always PDI-negative. Focal type I cell-like formation of epithelial cells occured in severely injured tissue (Fig. 4d, 5d).

Discussion

The activity of the PDI as subunit of prolyl 4-hydroxylase can be related to various catalytic functions. First, the PDI contributes to the catalytic sites of prolyl 4-hydroxylase and may be responsible for retaining the prolyl 4-hydroxylase tetramer in the ER [26]. There is, however, controversy as to whether prolyl 4-hydroxylase activi-

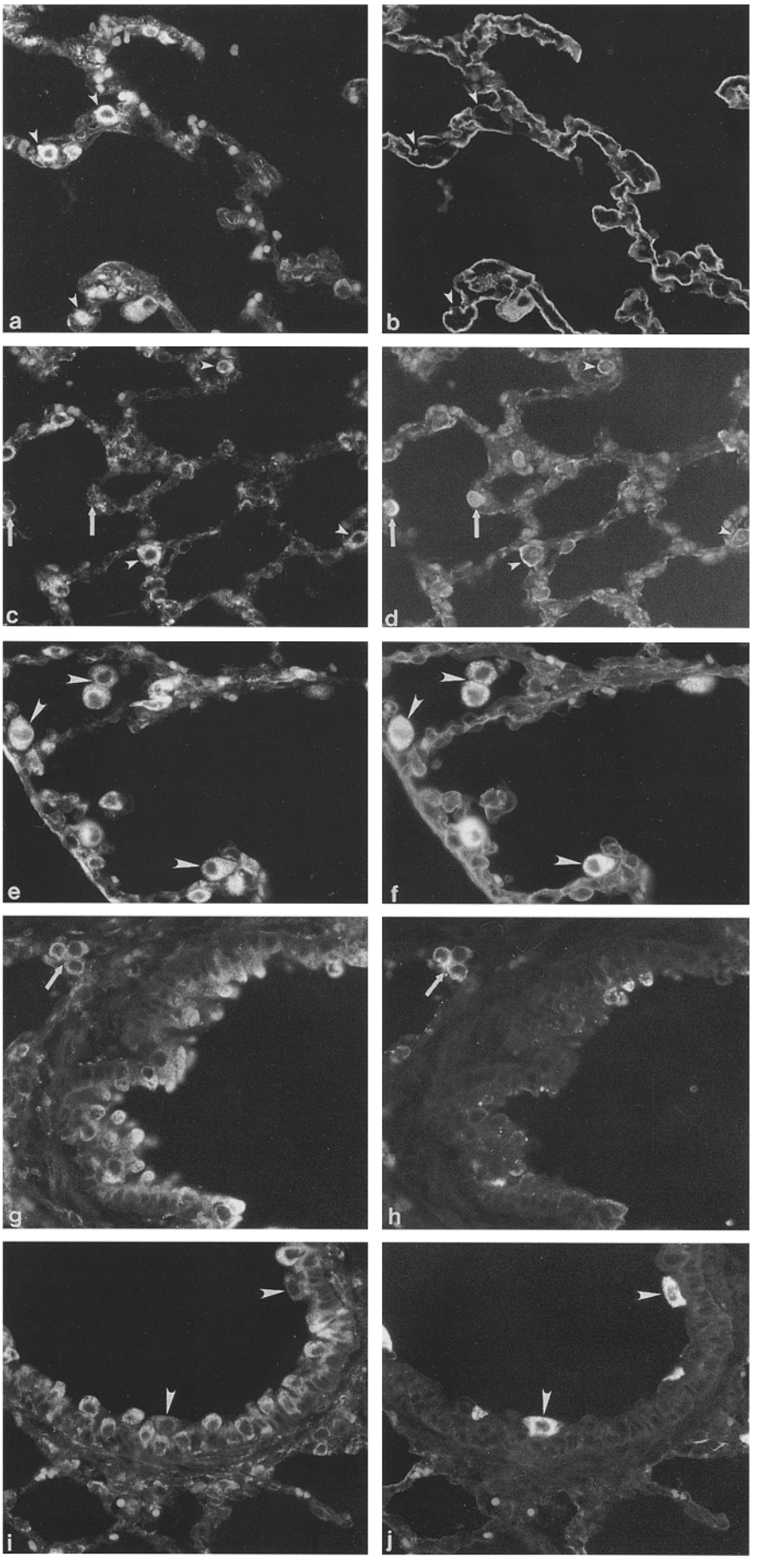

Fig. 3a–j Normal rat lung. Paraffin section. Double immunofluorescence demonstration of PDI (**a, c, e, g, i**) with BPA lectin (**b**), with cytokeratin (**d**), with MPA lectin (**f**), with SP-A (**h**), and with DBA lectin (**j**). Note the gaps in BPA staining at places of PDI-positive type II cells (*arrowheads in* **a–b**). Some autofluorescent erythrocytes also occurred. **c–d** Circular cytokeratin pattern of type II cells (*arrowheads*) and globular cytokeratin pattern of alveolar brush cells (*arrows*), which are PDI-negative. **e–f** MPA binding to PDI-positive alveolar macrophages (*arrowheads*). The usually apical MPA-binding typical for type II cells is not seen in this preparation. **g–h** Bronchial epithelium with apical PDI reactivity (**g**) and weaker SP-A staining (**h**). Note three type II pneumocytes in the left upper corner (*arrow*). **i–j** DBA-binding alveolar macrophages with low immunoreactivity for PDI (**j**, *arrowheads*; compare the strong reactivity of MPA-binding alveolar macrophages in **f**), **a–j** 350

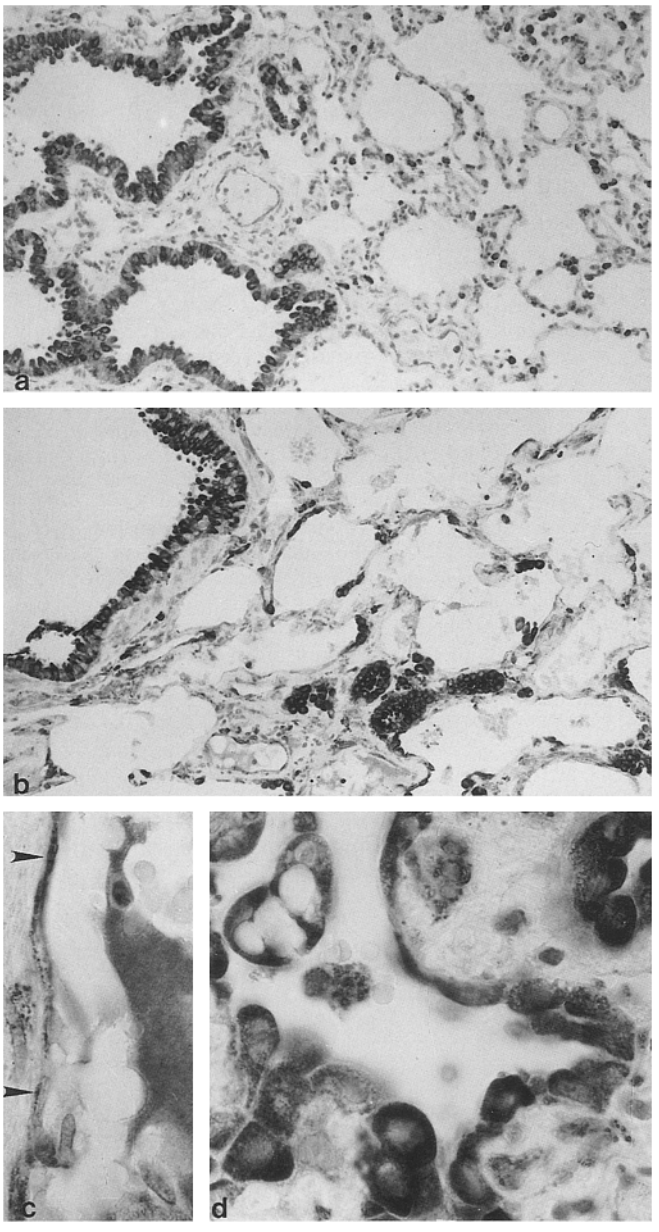

Fig. 4a–d Mini pig lung. Paraffin section. PDI immunostaining (1D3 antibody) of normal tissue (**a**) and of irradiated samples, 5×6 Gy group (**b**) and 5×8 Gy group (**c, d**). Note the strong staining of Clara cells and type II pneumocytes in **a. b** Strong reactions are detectable in hyperproliferative type II pneumocytes and weaker in the interstitium. **c** PDI staining of the endothelium (arrowheads) of a larger blood vessel. **d** Flattened and cuboidal alveolar epithelial cells with strong PDI immunoreactivity. ABC technique with weak Mayer's haematoxylin counterstain. **a–b** ×175; **c–d** ×550

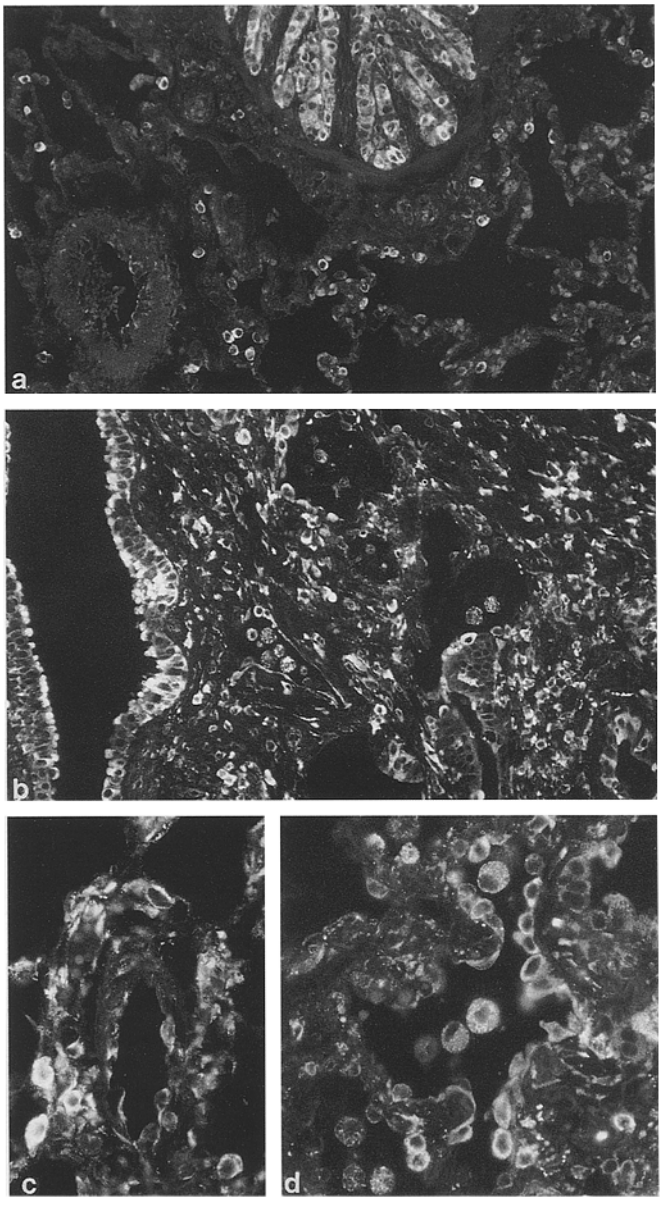

Fig. 5a–d Mini pig lung. Paraffin section. Comparative demonstration of PDI immunoreactivity by immunofluorescence technique. **a** Normal tissue. **b–d** Pulmonary fibrosis after irradiation (5×8 Gy group). **b** Note the strong staining of both the interstitial as well as the epithelial cells. **c** Enhanced PDI immunoreactivity in blood vessel endothelium. **d** Similar area as shown in Fig. **4d** with hyperplastic type II pneumocytes. **a–b** ×175; **c–d** ×350

ty needs PDI activity [11, 22, 31]. Other catalytic functions have been reported for the PDI as a cellular thyroid binding protein, as a component of the microsomal triglyceride transfer protein complex and as a glycosylation site binding protein of oligosaccharyl transferase [20, 33]. The complex changes during fibrogenesis which include increased production of extracellular matrix components and increased release of cytokines after activation of fibroblasts, alveolar macrophages and other inflammatory cells, prolyl 4-hydroxylase activity may be of particular importance in the persistance of the disease process. Cytokines involved in interstitial lung disease such as TGFβ and TNFα significantly increase the production of prolyl 4-hydroxylase [18]. Furthermore, 4 hydroxyproline has been found in collagens and in collagen-like domains of C1q subcomponent of complement,

of surfactant apoproteins SP-A and SP-D, of mannose-binding proteins, and of macrophage scavanger molecules [26]. These proteins are also found in abundance in fibrotic tissues in comparison with normal pulmonary tissue. Crouch et al. [5, 6] reported increased accumulation of extracellular SP-D in silica-induced pulmonary lipoproteinosis and in human alveolar proteinosis. They found intense immunoperoxidase staining of hypertrophic pneumocytes and bronchiolar cells for SP-D. Higher SP-A mRNA level in hyperplastic, reactive type II cells has been described previously by Broers et al. [3].

Interstitial fibrosis also involves increased collagen and other extracellular matrix protein synthesis [4, 28, 32]. Enhanced synthesis of collagen type IV has been implicated in fibrosis and other biological situations that imply cell migration, remodelling of the extracellular matrix, or injury to basement membranes [24, 25, 27].

Finally, macrophages play a central role in the pathogenesis of lung injury. They contribute to lung disease when activated [2, 23] and the consequences of such activation includes elevated expression of mannose-binding proteins and scavenger receptors [7, 23].

The present study demonstrates an increased number of PDI-immunopositive cells in fibrotic lung tissue. The early immunoreactivity of the capillary endothelia in this study is in line with the early activation of other endothelial and epithelial enzymes involved in the generation of oxy-radicals in radiation-induced pulmonary fibrosis in rats [9] as an example. Surprisingly, the PDI showed greatest immunoreactivity in the epithelial cells lining thickened septa (presumably regenerating type II cells). Possible explanations are the specific up-regulation of the PDI for enhanced surfactant protein synthesis (see above) or a general reaction to epithelial cell damage during fibrogenesis. The questions thus arises of whether the immunoreactivity for the β subunit of prolyl 4-hydroxylase can be related to one or more catalytic functions of the PDI in lung cells [8]. In view of the lack of data on the interaction of the alpha with the beta subunit in collagen synthesis, further studies are required to localize the alpha subunit in parallel. This is essential for the hydroxylation reaction in collagen metabolism [12].

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