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Immunohistochemistry of new type I alveolar epithelial cell markers of the rat

M. Kasper[1], M. Takeya[2], K. Takahashi[2], H. Grossmann[1], D. Schuh[1] and M. Müller[1]
[1]Institute of Pathology, Medical Faculty «Carl Gustav Carus», Technical University of Dresden, Dresden, Germany and
[2]The Second Department of Pathology, Kumamoto University, School of Medicine, Kumamoto, Japan

Summary. The presence of pan-cadherin and the MEP-1 antigen in normal and diseased rat lung was established by employing immunoperoxidase and double label fluorescence techniques. The binding of a mouse monoclonal antibody (MEP-1) reacting specifically with type I pneumocytes was assessed on paraffin sections of normal specimens as well as those with pulmonary fibrosis induced by bleomycin or radiation treatment. In injured alveolar epithelium, a diminished type I cell and a focal type II cell immunoreactivity was found. Electron microscopy of immunogold-labelled lung tissue confirmed the type I cell specificity of MEP-1. In severely injured pulmonary parenchyma MEP-1-negative areas occurred, which were also negative with the type II pneumocyte marker *Maclura pomifera* lectin. Similarly, a polyclonal pan-cadherin antibody uniformly decorated luminal surfaces of alveoli except the type II pneumocytes. Furthermore, pleural mesothelial cells, bronchiolar epithelial cells, endothelial cells of large blood vessels and alveolar macrophage surfaces exhibited pan-cadherin immunoreactivity. After injury, a remarkable loss of pan-cadherin immunoreactivity in the MEP-1-positive type I epithelial cells was detectable. These findings suggest that characterization of normal alveolar epithelial cells and monitoring of the epithelial remodelling in pulmonary pathohistology are sufficiently described by the antibodies MEP-1 anti pan-cadherin.

Key words: Alveolar epithelium, Rat lung, Monoclonal antibody, MEP-1, Pan-cadherin

Introduction

Pulmonary tissue comprises about 40 different cell types (Dormans, 1983). They have different morphology, function and histogenesis and they express various different molecules on the surface or in the cytoplasm. Despite the specific, often unknown, biological function, these molecules have been used as cell markers, which allow the study of the distribution of different pulmonary cell types in foetal development, in pathologically-altered tissue and in in-vitro experiments (Funkhouser and Peterson, 1989; Kasper and Singh, 1995).

For the characterization of the rat alveolar epithelium diverse lectins and cytoskeletal proteins have been successfully applied (Kasper and Singh, 1995). Type II pneumocytes are easily distinguishable by their cytokeratin (CK) pattern (Paine et al., 1988; Woodcock-Mitchell et al., 1990; Kasper et al., 1993a) as well as by their *Maclura pomifera* agglutinin (MPA) binding (Marshall et al., 1988; Kresch et al., 1991). Alveolar brush (type III) cells are selectively decorated with cytokeratin 18- and villin-specific antibodies (Höfer and Drenckhahn, 1992; Kasper et al., 1994a). Type I pneumocytes bind specifically *Lycopersicon esclulentum* agglutinin (LEA), *Erythrina cristagalli* agglutinin (ECA) or *Bauhinia purpurea* agglutinin (BPA) (Taatjes et al., 1990; Bankston et al., 1991; Kasper et al., 1994b). In addition, ICAM-1-specific antibodies are a type I cell marker (Christensen et al., 1993; Kang et al., 1993).

In this paper, we describe the pulmonary tissue reactivity of a monoclonal antibody (MEP-1) raised against a rat malignant fibrous histiocytoma (MFH) (Tsuchiya et al., 1990, 1993), which recognizes fibroblast-like cells of MFH. Furthermore, we studied the distribution of MEP-1 immunoreactive cells in an experimental rat model of pulmonary fibrosis (Kasper et al., 1993b,c, 1994c) and compared the MEP-1 immunoreactivity with the distribution of pan-cadherin using a polyclonal anti pan-cadherin antibody specific for the carboxy terminus of cadherins (Geiger et al., 1991; Kartenbeck et al., 1991). The pan-cadherin antibody stains type I pneumocytes and alveolar macrophages in human, mouse and mini pig pulmonary tissues (Kasper and Müller, 1994; M. Kasper, unpublished results).

Offprint requests to: Dr. Michael Kasper, Institute of Pathology, Medical Faculty Carl Gustav Carus, Technical University of Dresden, Fetscherstr. 74, D-01307 Dresden, FRG

Materials and methods

Tissues and immunocytochemistry

Samples of normal (n=10) and injured (n=10) pulmonary tissues were taken from previous studies (Kasper et al., 1993c,d, 1994b, 1995). As described before, paraffin sections were dewaxed and immunostained by using a commercially available ABC kit (Vector Laboratories, Fa. Camon, Wiesbaden, FRG) and diaminobenzidine as the developing agent. Wistar rats (n=5) were instilled intratracheally with 7 units of bleomycin sulphate, and Fischer rats (n=5) were exposed to a single radiation dose of 20 Gy (ultrahard X-ray). Irradiated animals were sacrificed at days 2, 14 and at weeks 4, 8 or 24 post-treatment. Bleomycin-exposed rats were sacrificed 6 weeks after treatment.

For double label fluorescence experiments the MEP-1 antibody was used in combination with biotinylated *Dolichos biflorus* agglutinin (DBA), biotinylated BPA, both 1:100 diluted (Vector Laboratories), or with MPA (FITC-labelled; dilution 1:100; Medac, Hamburg, FRG). Furthermore, rabbit polyclonal antisera directed against surfactant protein A (SP-A), dilution 1:20 (a kind gift from Dr. K. Sakai, Tokushima, Japan) and pan-cadherin (Sigma, 1:100 diluted) were included.

As secondary conjugates we employed goat anti mouse (for the detection of the monoclonal antibody MEP-1) and goat anti-rabbit (for the detection of the polyclonal antisera) immunoglobulins, FITC or Texas Red-coupled, dilution 1:80 (Dianova, Hamburg, FRG) and Texas Red-labelled avidine (Vector Laboratories, dilution 1:200).

Immunoelectron microscopy

Ultrathin sections of LR Gold-embedded rat lungs (for details see Kasper et al., 1994c) were incubated with 1:100 diluted monoclonal antibody MEP-1 overnight at 4 ºC. As secondary antibody we employed a goat anti-mouse Ig adsorbed to colloidal gold of 10 nm in size (Dianova), dilution 1:50. Washing was performed in TRIS-buffered saline. Grids were stained with 2% uranyl acetate for 3 min and counterstained with 1% lead citrate and examined in a Zeiss EM 900 electron microscope at 80 KV.

Controls for immunohistochemistry included omission of the primary antibody. Lectins were blocked by a 0.2M solution of the corresponding sugar N-acetylgalactosamine. Rat organs (liver, tongue, oesophagus, stomach), Schaffer-fixed, taken from our paraffin section library were used as the positive control for demonstration of pan-cadherin.

Immunoblotting

Lungs of adult Fischer rats with and without radiation-induced pulmonary fibrosis were homogenized in PBS containing 1 mM EDTA and 2 mM Pefabloc SC (Merck, Darmstadt, Germany). After addition of 5% Nonidet P40 (Sigma, Deisenhofen, Germany) the specimens were extracted overnight in the cold. The supernatants obtained after centrifugation were supplemented by 2.5% sodium dodecyl sulphate (SDS) and 5% 2-mercaptoethanol and were boiled for 5 minutes.

Electrophoresis was performed on an 8-16% Tris glycine gradient polyacrylamide gel (Novex, San Diego, Ca, USA) followed by semi-dry electrophoretic transfer onto a cellulosenitrate membrane (BA85, Schleicher and Schuell, dassel, Germany). After blocking with a 3% solution of nonfat dry milk the proteins were detected with the mab MEP-1 and peroxidase-conjugated anti-mouse IgG (H+L) (Dianova, Hamburg, Germany). Peroxidase activity was visualized with diaminobenzidine and nickel enhancement.

Results

Normal rat lung

The antibody MEP-1 stained all cells lining the alveolar spaces, except the type II pneumocytes (Fig. 1a,c,e, 4b). Double fluorescence staining employing type I cell-specific lectin BPA (Kasper et al., 1994b) revealed the type I cell specificity (Fig. 1c,d). Type II pneumocytes (MPA-positive, not shown; SP-A immunoreactive, Fig. 1e,f) and alveolar macrophages (DBA and BPA binding cells (Kasper et al., 1993b, 1994b), Fig. 1a-d) appeared negative. No staining of bronchial epithelial cells (not shown) and mesothelial cells (Fig. 3a) with antibody MEP-1 was found. Immunoelectron microscopy confirmed this selective reaction of the MEP-1 antibody (Fig. 2a,b). The polyclonal anti pan-cadherin antibody selectively decorated type I cells, alveolar macrophages and endothelial cells of large blood vessels (not shown). Further immunoreactions were found on mesothelial (Fig. 3b) and bronchial epithelial cells (not shown).

MEP-1 and pan-cadherin labelling in bleomycin- or radiation-exposed rat lungs

In all fibrotic samples studied, a focal or weaker expression of the MEP-1 antigen occurred on the surface of type I alveolar epithelial cells (Fig. 3a,c). At some sites, focal MEP-1 immunoreactivity was seen in alveolar macrophages (Fig. 4d). In severely injured areas of irradiated lungs (6 months after irradiation) and in bleomycin-injured lungs focal loss of MEP-1 reactivity was detectable (Fig. 4f). Double fluorescence staining with MPA, a well-known type II pneumocyte marker (Kresch et al., 1991), revealed that the MEP-1-negative areas were not replaced by type II pneumocytes (Fig. 4e,f). In single cases, focal MEP-1 reactivity occurred at the surface of type II pneumocytes (Fig. 4e,f). The SP-A expression pattern could no longer be used for the

selective demonstration and characterization of type II pneumocytes, because of the increased intraalveolar accumulation of SP-A in fibrotic specimens (not shown).

Staining with antibody to pan-cadherin was focal and of variable intensity (Fig. 3b,d). This type of antigenic alteration could be detected at early stages of the fibrotic process (about 4 weeks after treatment). MEP-1/pan-cadherin double staining revealed a focal loss of immunoreactivity in the epithelial cells lining the alveoli (Fig. 4c,d). Proliferative type II pneumocytes exhibited no pan-cadherin immunoreactivity (not shown).

In immunoblots of normal and irradiated specimens (tissue extracts of bleomycin-cases were not included in this study) MEP-1 detected a single protein band of 35 kD in comparison with the molecular weight standard stained with silver (Fig. 5) in accordance with Tsuchiya et al. (1993).

Discussion

MEP-1 reacts with fibroblast-like cells and certain epithelial cells of the rat, such as glomerular epithelial cells in the kidney, alveolar epithelial cells of the lung, and basal cells in the epidermis (Tsuchiya et al., 1990). Blood monocytes, alveolar and peritoneal macrophages were found to be negative. Immunoblotting has revealed that MEP-1 stains a protein band of 35 kD, but the exact nature of the antigen is not yet known (Tsuchiya et al., 1993).

The present investigation demonstrates a specific binding of monoclonal antibody MEP-1 to rat type I pneumocytes. In pulmonary fibrosis the degree of MEP-1 immunoreactivity with type I pneumocytes varied and focally extended to type II cells. Therefore we presume that the stability of the lectin MPA as type II cell marker continued in the reepithelialization process. There is, however, some evidence for a changing membrane protein or carbohydrate composition on the surface of pathologically altered pulmonary epithelial cells (Taatjes et al., 1991; Spicer and Schulte, 1992; Sugiyama et al., 1992; Sugiyama and Kawai, 1993; Kasper et al., 1993d; Meyer et al., 1993). Thus, the focal positivity

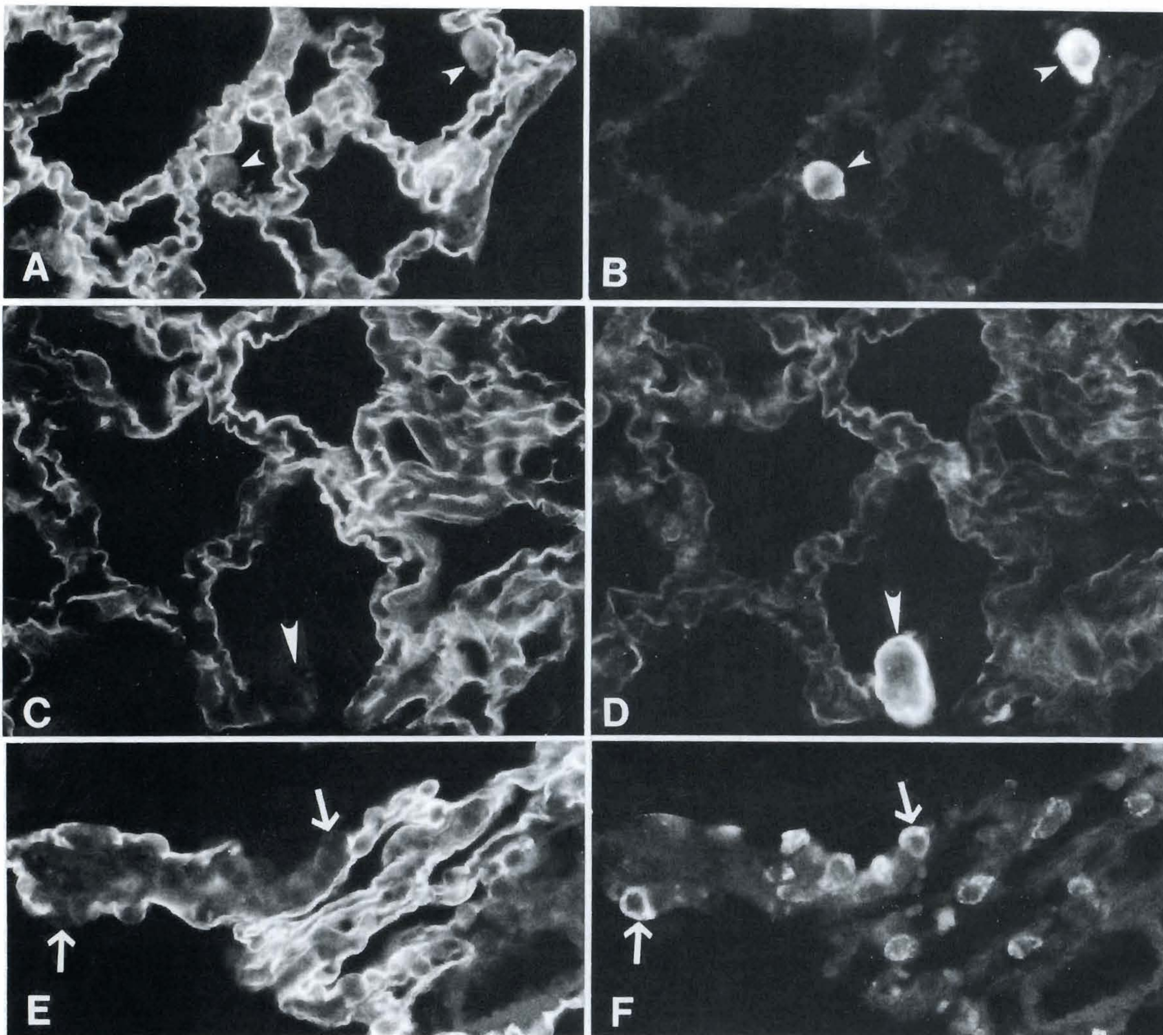

Fig. 1a-f. Normal rat lung. Paraffin sections. Double label fluorescence for the simultaneous demonstration of monoclonal antibody MEP-1 **(a, c, e)** and lectins DBA **(b)**, BPA **(d)** and surfactant protein A **(f)**. Alveolar macrophages seen in b and d (arrowheads) and type II cells stained by polyclonal anti SP-A antiserum (f, arrows) are devoid of MEP-1 reactivity. Type I alveolar epithelial cells bind BPA (d) and are MEP-1 positive (a, c, e). At some sites, type II pneumocytes are covered by type I cells, thus mimicking a MEP-1 positivity of type II pneumocytes. x 300

of type II cell clusters does not justify the MEP-1 antibody as a marker of type II cell injury. It seems more likely that the antigen recognized by MEP-1 is a differentiation-related characteristic of type I cells, which can be induced when type II pneumocytes undergo transformation into type I cells. Nevertheless, the MEP-1 antibody can be regarded as a further tool for selective visualization of type I pneumocytes. The main advantage of the usage of MEP-1 to specify type I pneumocytes is its application to electron microscopy and its easy application to various double stainings with polyclonal antibodies or with diverse lectins.

A second interesting finding of the present study is the loss of an antigen recognized by a polyclonal anti pan-cadherin antiserum on type I cells of normal alveolar epithelium. In contrast to type II pneumocytes, type I pneumocytes show a remarkable abundance in adhesion molecules such as ICAM-1 (Christensen et al., 1993; Kang et al., 1993) and, as shown here for the normal lung, in pan-cadherin immunoreactivity. Whereas the biochemical properties of type II cells are largely explored, just a few type I specific antigens are known (Dobbs et al., 1988; Danto et al., 1992; Hotchkiss et al., 1992; Nagae et al., 1993; for type I-specific lectins see above). The presence of pan-cadherin immunoreactivity on alveolar epithelial cells need further confirmation by rat specific E-cadherin antibodies. The first investigations employing an anti-dog-E-cadherin antibody, a kind gift from Dr. Behrens, Berlin, Germany, and a rat reactive anti-mouse E-cadherin antiserum (kindly provided by Dr. R. Kemler, Freiburg, Germany) demonstrated a focal type I pneumocyte as well as a basolateral type II pneumocyte staining (M. Kasper, unpublished results). In normal human lungs, E-cadherin can be selectively localized in type II pneumocytes, whereas other epithelial adhesion molecules have been localized at the surface of the entire alveolar epithelium (Kasper et al., 1995).

In summary, type I alveolar epithelial cells of the rat can be selectively decorated by the monoclonal antibody MEP-1 and the polyclonal anti pan-cadherin antiserum. Even though their immunoreactivities are not unique to type I pneumocytes, both reagents can successfully be used as a marker for these cells in the study of their development and biology and for the characterization of the alveolar epithelial remodelling in pulmonary fibrosis.

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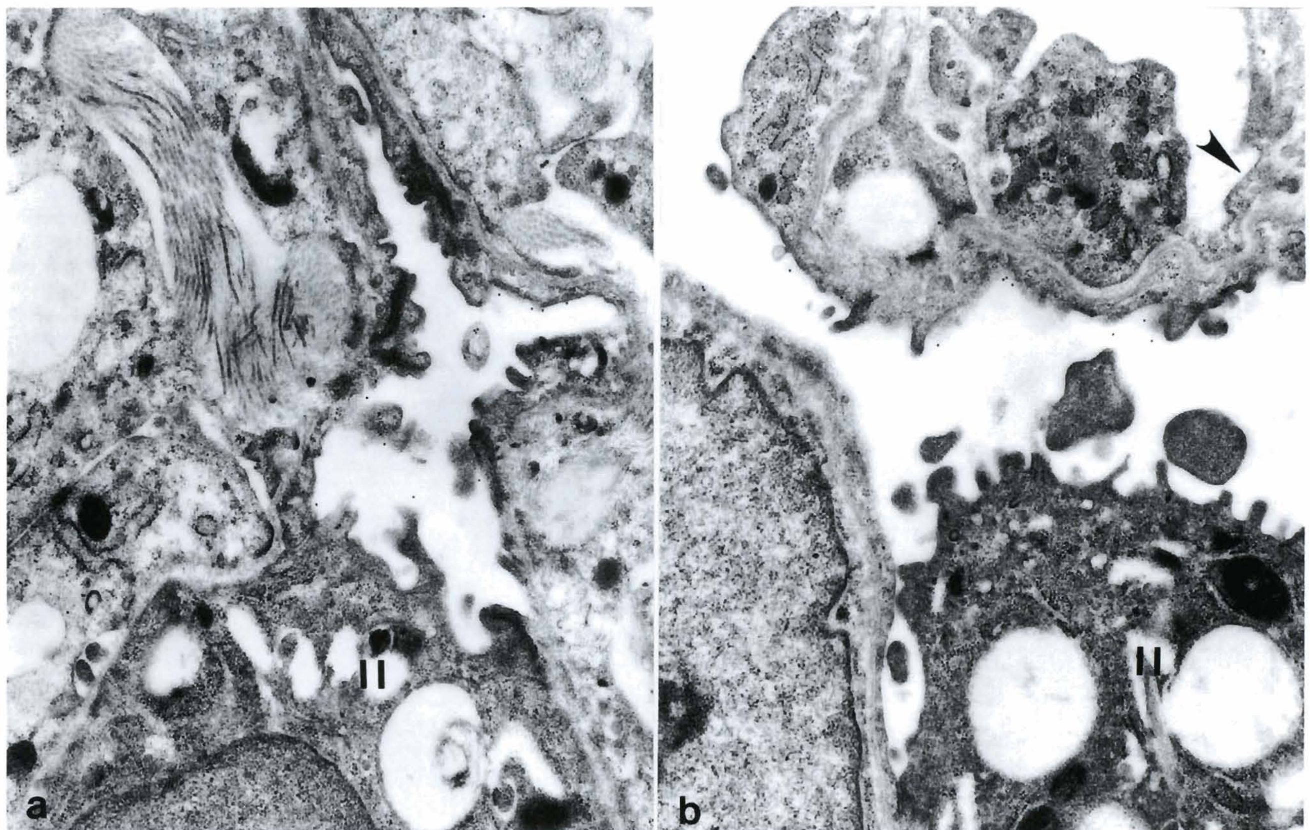

Fig. 2. a-b. Immunogold labelling of LR Gold embedded rat lung. Primary antibody MEP-1. Note the presence of gold particles on type I cells. On the type II cell (II) and the capillary endothelium (b, arrowhead) no gold labelling occurred. x 18,150

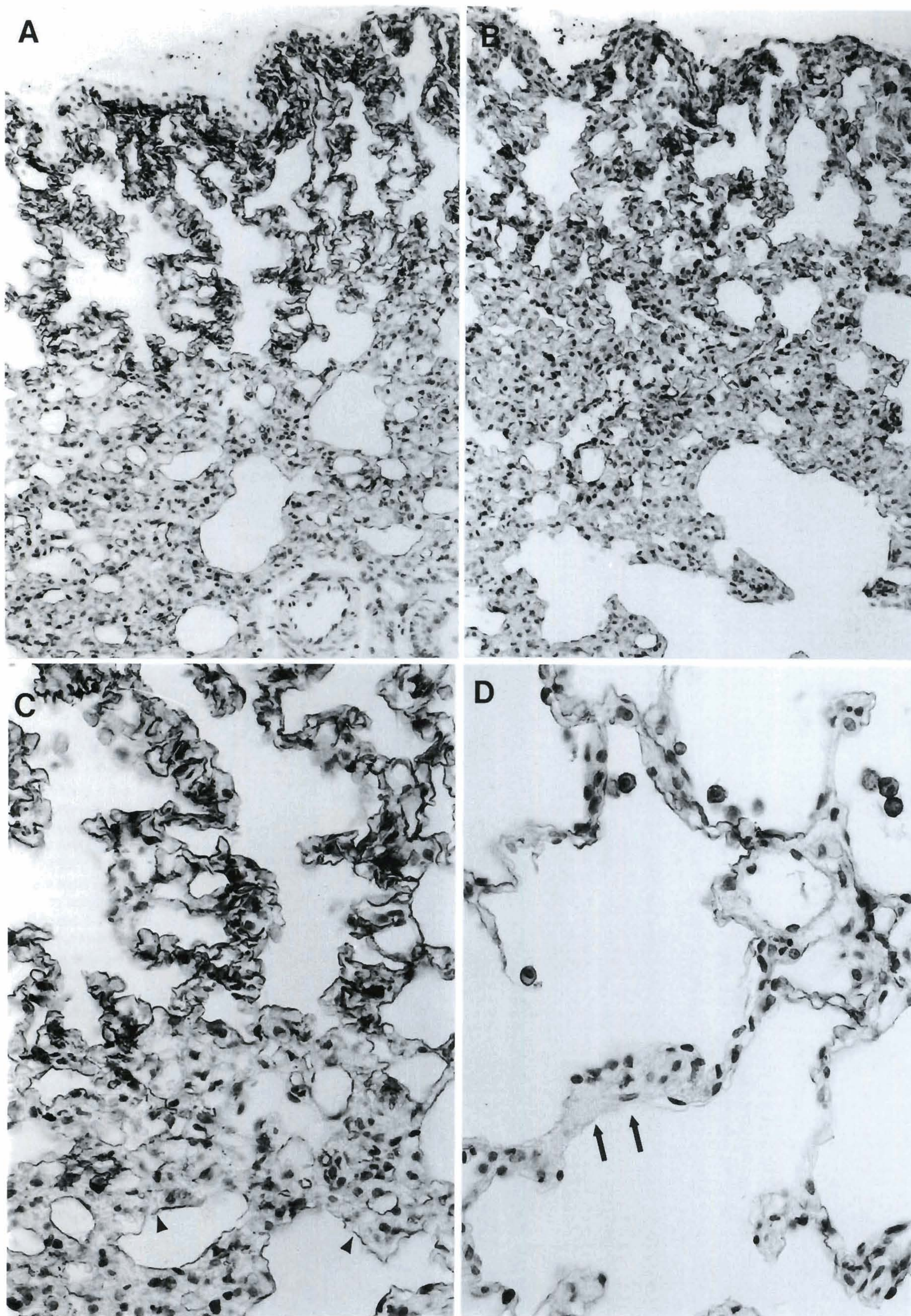

Fig. 3. Radiation-induced fibrosis. Paraffin sections. Immunoperoxidase demonstration of MEP-1 (**a, c**; c higher magnification of a) and pan-cadherin (**b,d**). Note the gradual loss of MEP-1 and pan-cadherin immunoreactivity from the subpleural «normal» region towards the fibrotic area. Thickening of alveolar wall is accompanied by a lower degree of MEP-1 staining (arrowheads, c) and a loss of pan-cadherin immunoreactivity (d, arrows). a, b x 150; c, d x 300

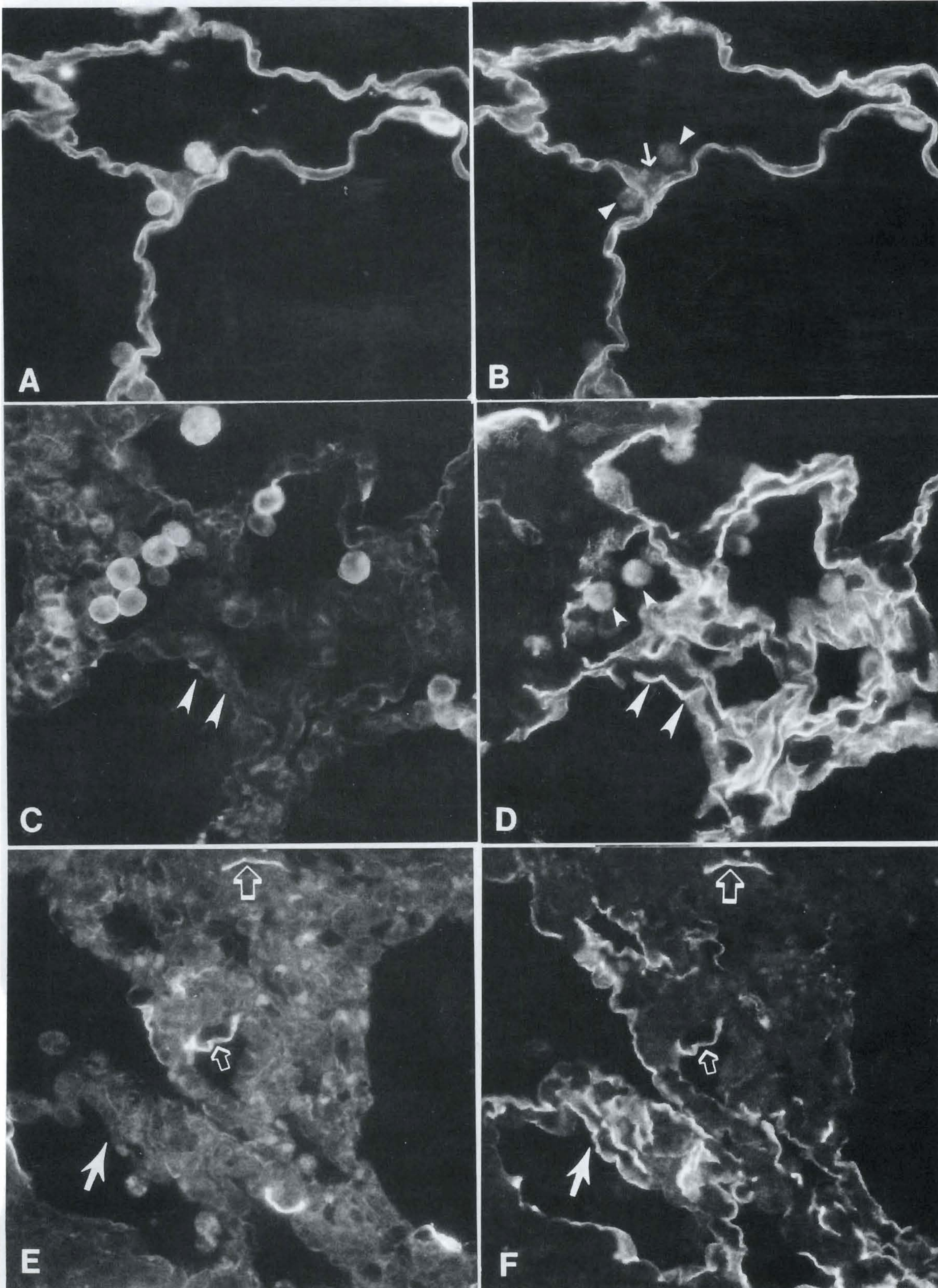

Fig. 4. Bleomycin-induced pulmonary fibrosis (8 weeks after exposure). **a, b.** «Normal» area near the pleura with colocalization of pan-cadherin (a) and MEP-1 (b) in type I cells. b. A type II pneumocyte (arrow) and alveolar macrophages (arrowheads) are MEP-1 negative. **c-f.** Fibrotic area double-stained for pan-cadherin (c) or MPA lectin (e) and MEP-1 (d, f). Note the presence of MEP-1 reactivity in pan-cadherin negative alveoli (c, d, arrowheads), which are not replaced by type II cells (compare the MPA/MEP-1 double staining; arrows in e, f). Open arrows in e, f indicate $MPA^{+}/MEP\text{-}1^{+}$ type II cells. d. Note a weak MEP-1 staining of alveolar macrophages (d, small arrowheads). x 300

Fig. 5. Immunoblot of rat lung extracts without (lane 2) and with radiation-induced pulmonary fibrosis (lanes 3 and 4, in lane 4 the antibody MEP-1 was omitted. Lane 1: molecular weight markers (Novex, San Diego, Ca, USA)). MW in kD from the top to the bottom: 200, 116, 97, 66, 55, 36, 31, 20.

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