Virchows Arch (1999) 435:20–31

ORIGINAL ARTICLE

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Alterations in the immunohistochemical distribution patterns of vascular endothelial growth factor receptors Flk1 and Flt1 in bleomycin-induced rat lung fibrosis

Received: 27 August 1998 / Accepted: 31 March 1999

Abstract To investigate the role of vascular endothelial growth factor (VEGF) in fibrogenesis, the distribution patterns of the VEGF receptors Flt1 and Flk1 were studied by immunohistochemistry, double immunofluorescence, and immunoelectron microscopy in normal (n=2) and bleomycin-treated (n=21) adult rats. Lungs were studied at 5, 24, 28, 35, and 42 days after treatment (p.t.). Flt1, Flk1, and VEGF immunoreactivity localised predominantly to the pulmonary epithelium. In control lungs, Flt1 immunoreactivity was present in ciliated bronchial epithelium and type 2 pneumocytes, Flk1 in Clara cells, and VEGF in Clara cells and type 2 pneumocytes. Flk1 localised to mast cells, present in the peribronchovascular and pleural interstitium only. Flt1- and Flk1-mRNAs were observed in Clara cells and type 2 pneumocytes. Bleomycin-induced fibrogenesis was characterised by a decrease in Flk1 immunoreactivity of Clara cells, and an increase in VEGF-immunoreactive myofibroblasts and type 2 pneumocytes by day 5 p.t., followed by a progressive accumulation of Flk1-immunoreactive mast cells by day 24 p.t. in fibrotic lesions containing VEGF-immunoreactive myofibroblasts. After 42 days, fibrotic regions were densely populated by mast cells. Since mast cells are known to be chemotactically attracted by VEGF, we suggest that VEGF/Flk1 represents the molecular link between proliferation of myofibroblasts, accumulation of mast cells, and the burst of fibrosis at sites of initial lesions in bleomycin-induced fibrosis.

Key words Pulmonary fibrosis · Bleomycin · Alveolar epithelium · Bronchiolar epithelium · Vascular endothelial growth factor · VEGF

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Introduction

Pulmonary fibrosis can result from various types of injury to the lung. The pathogenesis is characterised by an initial acute inflammatory reaction, which may lead to a chronic fibroproliferative process. The pulmonary architecture is profoundly remodelled, with the extracellular matrix and a variety of cell types involved [8, 50]. The bleomycin-treated rat lung is an animal model of pulmonary fibrosis commonly used to investigate the underlying pathogenic mechanisms. Bleomycin is usually given intratracheally resulting in lung injury similar to that produced by the parenteral routes, but with the advantages of a quicker kinetic development and a clear initiation time for lung injury [50]. The characteristic structural and ultrastructural alterations have been described in detail [3, 31, 32]. Immunohistochemistry has been used successfully to dissect the complex changes that occur in time and space during fibrogenesis and to investigate the specific role of certain cell types [23, 24, 54, 56], transcription factors [18], and growth factors [58]. Recently, we have presented evidence that vascular endothelial growth factor (VEGF) may also be involved in bleomycin-induced pulmonary fibrogenesis [12].

VEGF has emerged as a cytokine with important functions in the regulation of angiogenesis associated both with normal embryonic development [5] and with tumorigenesis [10, 47]. Recent studies indicate that VEGF may also be involved in pathogenic events associated with hyperoxia [34], acute and chronic hypoxia [36, 52], and pul-

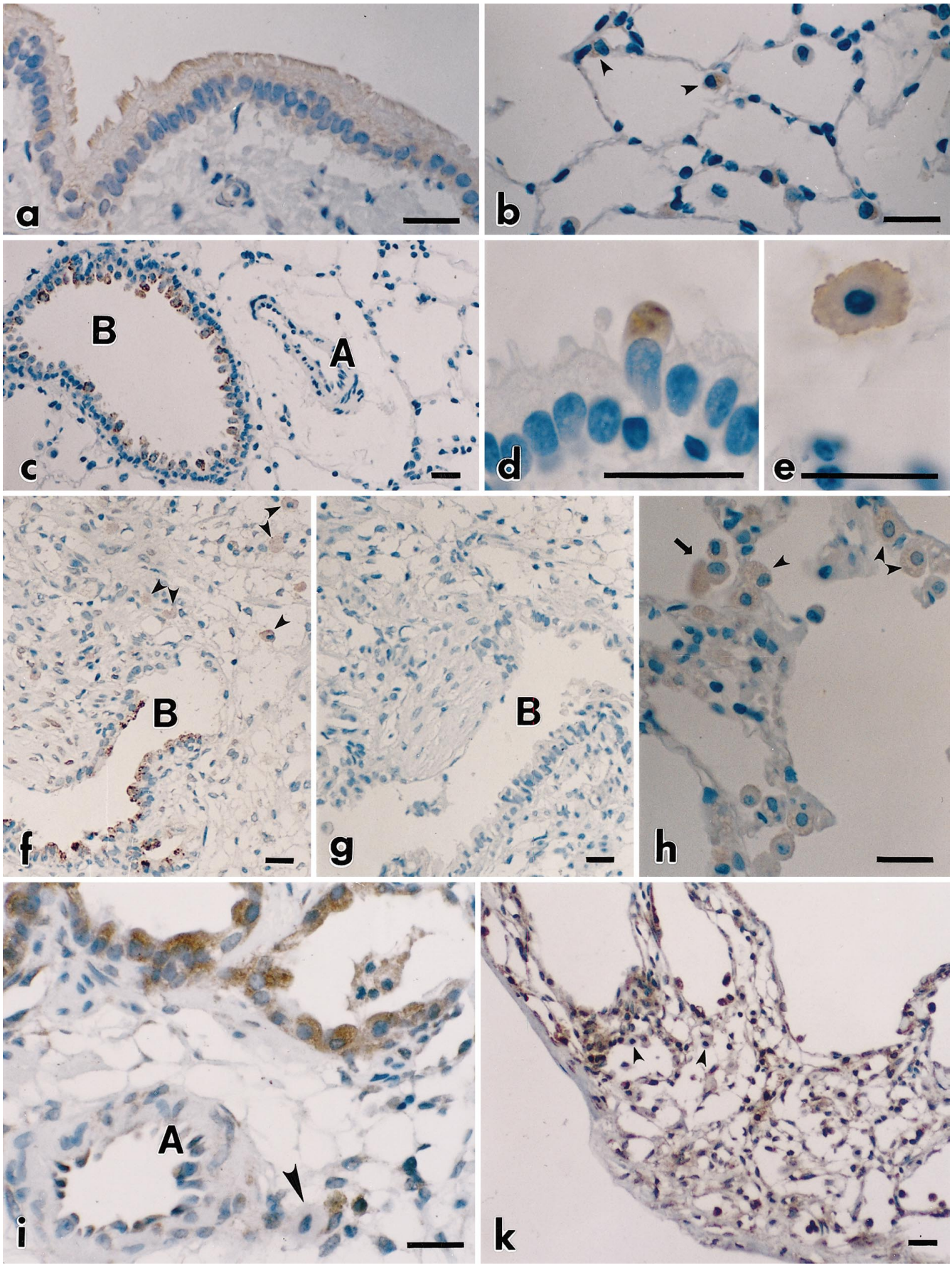

Fig. 1a–k

monary sarcoidosis [51]. Two high-affinity receptors of VEGF, today also termed VEGF-A, have been characterised, VEGFR-1 or Flt1 (fms-like tyrosine kinase) and VEGFR-2 or KDR (kinase insert domain region containing receptor), the murine homologue of which is Flk1 (fetal liver kinase) [13]. A third receptor, VEGFR-3 or Flt4, binds VEGF-C, which appears to be involved in lymphatic vessel development [22]. Flt1 binds VEGF and placental growth factor (PlGF), while Flk1 binds VEGF-A [38] and VEGF-B [41], which is thought to be involved in angiogenesis and endothelial growth in muscle [41]. The role of VEGF and its receptors in the regulation of angiogenesis has been comprehensively reviewed [40]. Evidence has been presented that a further member of the VEGF family, designated VEGF-D, may be another ligand of the receptor Flk1 [2]. The recently discovered member VEGF-E was found to bind with high affinity to Flk1, but did not bind to Flt1 [38].

To further explore the role of VEGF in the development of pulmonary fibrosis, we investigated the immunohistochemical distribution patterns of the VEGF receptors Flt1 and Flk1 in normal adult rat lung and in bleomycin-induced pulmonary fibrogenesis. Double immunofluorescence microscopy was used to characterise the cell types exhibiting immunoreactivity for Flt1 and Flk1, respectively. The mRNAs of Flt1 and Flk1 were localised by in situ hybridisation as an independent method to validate immunohistochemical results. Postembedding immunogold labelling was used to study the subcellular localisation of Flk1 and Flt1 by electron microscopy.

Materials and methods

The lungs examined were obtained from a previous study [12]. Briefly, female Wistar rats (200–300 g body weight) received a single intratracheal dose of 7 units of bleomycin sulphate dissolved in 250 µl of 0.9% NaCl. Rats were sacrificed at day 5 (n=3), 24 (n=5), 28 (n=4), 35 (n=4), and 42 (n=5) after treatment. Control lungs were obtained from two untreated rats.

For immunostaining the following primary antibodies were used: (a) affinity-purified polyclonal rabbit antibody raised against a peptide corresponding to amino acids 1312–1328 mapping at the carboxy terminus of the precursor form of human Flt, which exhibits no cross-reactivity with Flk1, Flt4 or other tyrosine kinase receptors (Santa Cruz Biotechnology, Santa Cruz, USA); (b) affinity-purified polyclonal rabbit antibody raised against a peptide corresponding to amino acids 1158–1345 mapping at the carboxy terminus of the precursor form of Flk1 of mouse origin, which exhibits no cross-reactivity with other protein tyrosine kinase receptors (Santa Cruz Biotechnology); the antibody recognises a single protein band at about 200 kDa consistent with the published molecular mass of Flk1 [33], (c) monoclonal mouse antibody against a peptide corresponding to amino acids 1–191 of VEGF of human origin with a deletion from amino acids 142–185, which is non-cross-reactive with VEGF-B, VEGF-C or PlGF (S. Cruz Biotech.). The antibodies against Flt1, Flk1, and VEGF were applied at dilutions of 1:80, 1:50, and 1:100 in incubation buffer, respectively.

For double immunofluorescence labelling the following secondary antibodies, lectins, and conjugates were used: (a) polyclonal mouse anti-rat alpha smooth muscle (α-SM) actin antibody (Dianova, Hamburg, Germany), diluted 1:50; (b) monoclonal mouse antibody against surfactant protein D (SP-D) of rat (Dr. S. Albrecht, Dresden, Germany, [26]), undiluted; (c) *Ulex europaeus* agglutinin (UEA) (Vector Laboratories, Burlingame, Calif.), diluted 1:10; (d) *Lycopersicon esculentum* lectin (LEL) (Vector Lab.), diluted 1:200; (e) *Maclura pomifera* agglutinin (MPA; Vector Lab.), diluted 1:100; (f) monoclonal mouse anti-human mast cell tryptase antibody AA1 (DAKO, Glostrup, Denmark), diluted 1:40; (g) anti-rabbit antibody coupled to 4,6-dichlorotriazinyl-aminofluorescein (DTAF; Dianova), diluted 1:200; (h) Texas red-coupled anti-mouse antibody (Dianova), diluted 1:80 for labelling of antibody against α-SM actin; (i) Texas red-coupled avidin (Vector Lab.), diluted 1:100 for labelling of biotinylated lectins UEA, LEL, and MPA; (k) for indirect immunoelectron microscopy, 20 nm gold-labelled goat anti-rabbit IgG (Dianova) was used to detect the primary rabbit antibodies.

As described previously [12, 27], lungs were perfused with phosphate-buffered saline (PBS, pH 7.4), fixed in 4% formaldehyde in PBS, and embedded in paraffin for subsequent immunohistochemical analysis. Additionally, cryostat sections of a control lung frozen in liquid nitrogen were used for double immunofluorescence labelling. For immunoelectron microscopy, tissue blocks were fixed in 4% paraformaldehyde and 0.05% glutaraldehyde in 0.1 M cacodylate buffer (pH 7.4), infiltrated with 2.3 M sucrose in PBS over night, frozen in liquid nitrogen, cryo-substituted at –80°C in methanol, and embedded in Lowicryl HM20.

As described in detail previously [12, 27], dewaxed, microwave-irradiated paraffin sections were incubated with the affinity purified primary antibodies at the appropriate dilution (see above) in PBS for 1 h at 37°C after blocking of endogenous peroxidase and unspecific staining. The primary antibodies were detected by peroxidase-coupled goat anti-rabbit IgG (HRP77; Dr. Grossmann, Dresden) or by means of the avidin-biotin peroxidase complex

◀ **Fig. 1** Immunohistochemical staining of **a**–**e** normal and **f**–**k** fibrotic rat lung to demonstrate the distribution of **a, b** Flt1, **c**–**h** Flk1, and **i, k** VEGF. Staining for Flt1 was seen in **a** ciliated bronchial epithelium, and in **b** type 2 pneumocytes (*arrowheads*) of alveolar epithelium. Staining for Flk1 was restricted to **c, d** nonciliated bronchiolar Clara cells, and **e** interstitial mast cells. With the onset of bleomycin-induced alterations at 5 days after treatment, **h** additional, albeit weak staining for Flk1 was seen in type 2 pneumocytes (*arrowheads*) and alveolar macrophages (*arrow*). In advanced fibrosis at 42 days after treatment, **f** fibrotic regions contained numerous Flk1 immunoreactive mast cells (*arrowheads*). Staining of adjacent section was completely abolished by **g** preabsorption of the Flk1-antibody with the control peptide offered by the manufacturer. The fibrotic areas were characterised by **i**, **k** the presence of numerous VEGF-positive interstitial fibroblasts that were in close association with mast cells (*arrowheads*); specimen obtained 42 days after bleomycin treatment (*A* artery, *B* bronchiole). *Scale bars* 20 µm

Fig. 2 Double immunofluorescence staining **a**–**d** of cryostat-sections of frozen tissue, and immunoelectron microscopic labelling **e, f** of ultrathin sections of cryosubstituted, Lowicryl-embedded tissue of normal adult rat lung. Sections were labelled with primary antibodies against **a, c, e, f** Flt1, **b** surfactant protein D (SP-D), or with **d** *Lycopersicon esculentum* lectin (LEL). Immunofluorescence labelling for Flt1 localised to **a** apical face of ciliated bronchiolar epithelium, while **b** Clara cells (*arrowheads*), which stained intensely for SP-D, were free of Flt1. In turn, **c** alveolar type 2 pneumocytes exhibited intense staining of cytoplasmic granules for Flt1, while **d** the LEL-labelled type 1 pneumocytes (*arrowheads*) were devoid of Flt1. Note the inverse staining intensities of type 2 pneumocytes (*P2*; *P2*[1]) for Flt1 and SP-D in **a** and **b**. Immunoelectron microscopy using secondary antibodies conjugated to **e** 20 nm- or **f** 5 nm-gold revealed that in type 2 pneumocytes cytoplasmic granular staining for Flt1 was associated with multivesicular bodies (*arrowheads*;. *Nu* nucleus). *Scale bars* **a**–**d** 20 µm, **e, f** 25 nm ▶

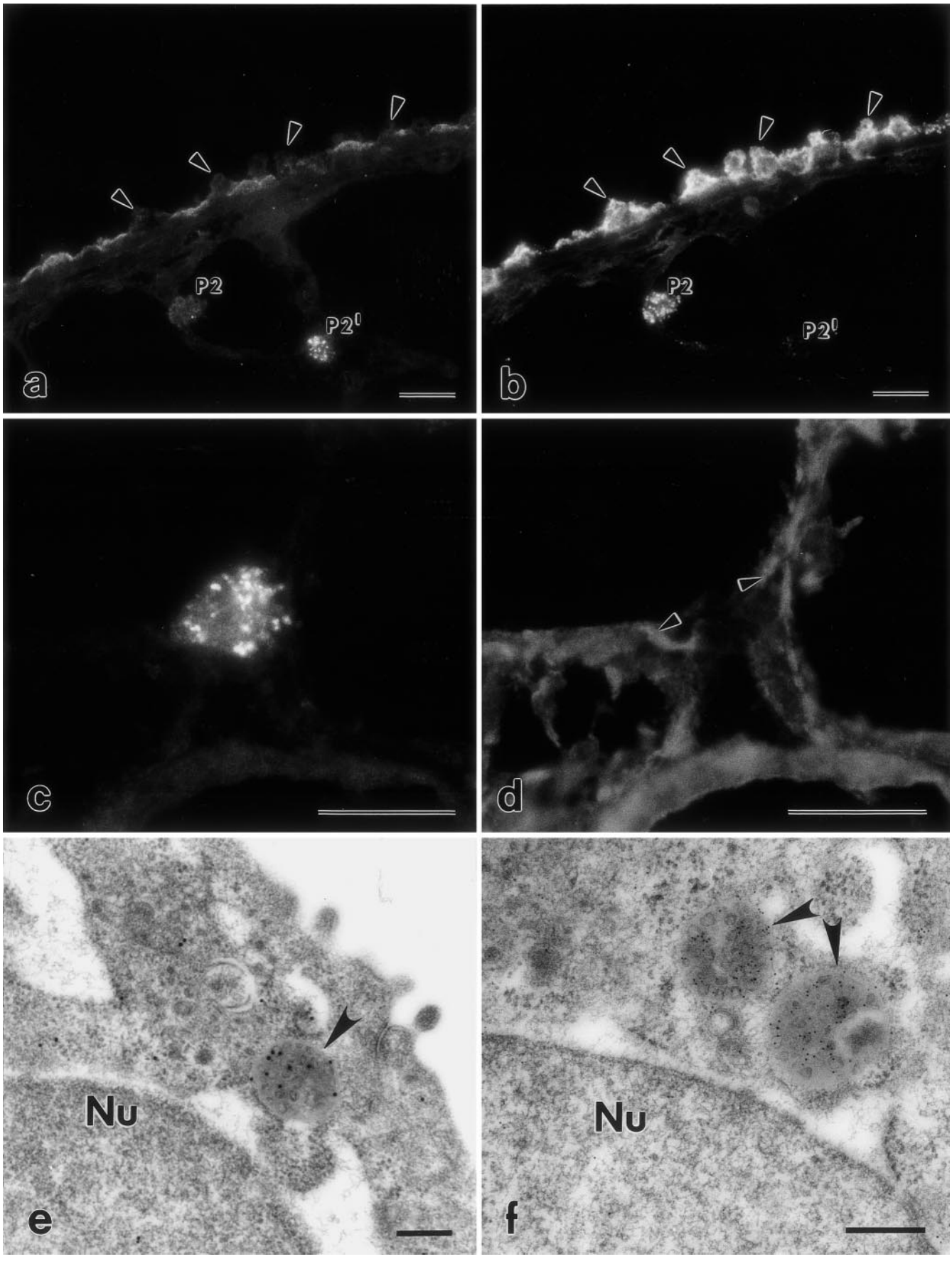

Fig. 2

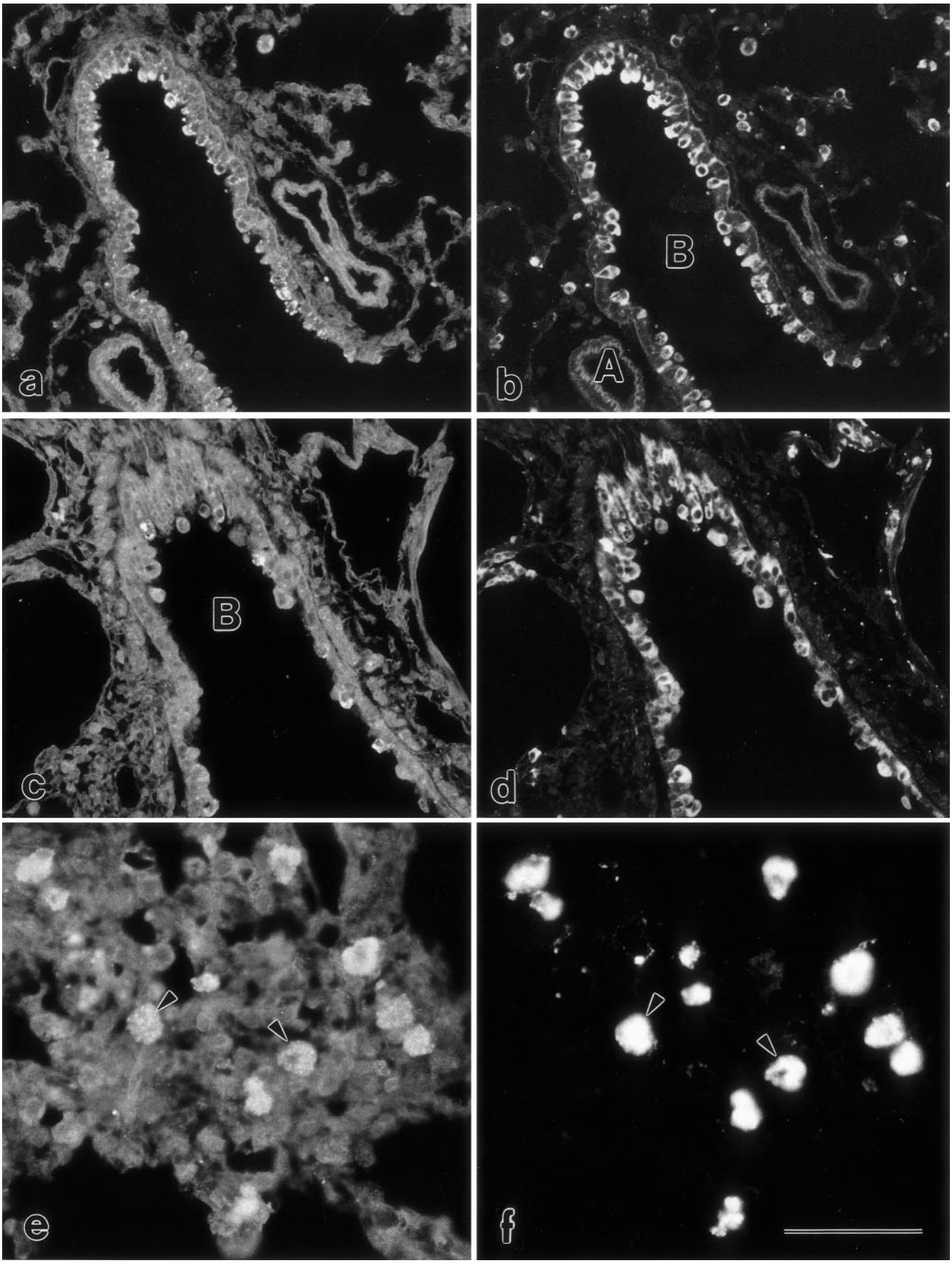

Fig. 3

(ABC) technique (Vectastain Elite kit, Vector Lab.). Sections were briefly counterstained with haemalaun.

Immunofluorescence labelling for Flt1 could only be performed on cryostat sections of frozen lung tissue. Since immunoperoxidase staining showed no alteration in the staining pattern with progressing fibrosis, double labelling was restricted to normal rat lung tissue. Immunofluorescence labelling for Flk1 was performed using formalin-fixed, paraffin-embedded material. Sections of normal rat lungs (n=2) and of experimental rat lungs fixed at 5 days (n=2), 24 days (n=2) and 6 weeks (n=2) after bleomycin treatment, respectively, were investigated. Dewaxed and microwave-treated sections or frozen sections were stained as described recently [12]. After blocking of unspecific background staining, sections were incubated with anti-Flk1 antibody (undiluted) or anti-Flt1 antibody (diluted 1:80) stained with DTAF-coupled goat anti-rabbit IgG (Dianova). Secondary antibodies or lectins (see above) used as cell type-specific markers [24] were stained with Texas red-coupled goat anti-mouse IgG (Dianova) or Texas red-coupled avidin (Vector Lab.).

To estimate changes in Flk1 labelling after bleomycin-treatment, two sections of two lungs per time point (control, 5, 24, 42 days p.t., respectively) were double labelled for Flk1 and SP-D. Starting from a random position outside the section, each section was scanned by moving the microscope stage at a constant distance along the x- and y-axes. At each position all bronchiolar epithelial cell profiles falling inside the counting frame or hitting the acceptance line and exhibiting one or both markers were counted [37]. The numbers of cell profiles exhibiting one or both markers were determined, and the relative contributions of the phenotypes $Flk1^+/SP\text{-}D^+$ and $Flk1^+/SP\text{-}D^-$ were calculated.

For immunogold labelling of Flt1 and Flk1, ultrathin sections were transferred to 200-mesh nickel grids coated with 3% collodion. After blocking of nonspecific labelling with 50 mM glycine and 2% gelatin, 0.5% acetylated BSA ultrathin sections were incubated overnight at 8°C with anti-Flt1 antibody (diluted 1:20) or with anti-Flk1 antibody (undiluted), both of which were detected with goat anti-rabbit IgG coupled to 20-nm gold particles (Dianova), diluted 1:50. Sections were counterstained with 2% uranyl acetate and Reynold's lead citrate, and examined with a Zeiss EM 900.

Specificity controls were performed, substituting the primary antibodies by primary antibody absorbed to the corresponding control peptide (Santa Cruz Biotechnology) and by omission of the primary antibody. The lectin binding was specifically blocked by the corresponding sugar (for details, see [24]).

Plasmid hFlt-1 (kindly provided by Dr. G. Breier, Bad Nauheim, Germany [43]) was linearised with NotI and transcribed with T3 RNA polymerase for production of a Biotin-labelled antisense probe. Plasmid pBluescript II KS +/- containing a 1 kb KDR-derived (nucleotides 715–1750) insert (kindly provided by Dr. B.I. Terman, Pearl River, N.Y. [49]) was linearised with XbaI and transcribed with T3 polymerase using the MAXIscript in vitro transcription kit (Ambion, Austen, Tex.) according to the manufacturer's instructions. In situ hybridisation was done with the DAKO GenPoint system using paraffin sections. Probe concentrations were 10 ng/µl. Hybridisation buffer was from DAKO Diagnostica (Hamburg, Germany).

◀ **Fig. 3** Double immunofluorescence staining of **a, b** normal and **c, d** fibrotic rat lung 24 days and **e, f** 42 days after treatment with bleomycin. Colocalization of **a, c** Flk1 and **b, d** SP-D showed a marked decrease in the number of SP-D labelled Clara cells co-expressing Flk1 in the fibrotic rat lung while immunoreactivity for SP-D appeared to be unchanged. Colocalization of **e** Flk1 and **f** mast cell tryptase demonstrated the presence of numerous Flk1-immunoreactive mast cells (*arrowheads*) characteristic of fibrotic areas (*A* artery, *B* bronchiole). *Scale bars* **a**–**d** 100 µm, **e, f** 50 µm

Results

Immunperoxidase staining of formalin fixed, paraffin embedded rat lung revealed differences in the distribution patterns of the VEGF receptors studied (Fig. 1). While staining for Flt1 was seen in both airways and parenchyma of the lung (Fig. 1a, b), immunoreactivity for Flk1 was detected in the airways only (Fig. 1c–e). In the pulmonary parenchyma, alveolar type 2 pneumoctyes were immunoreactive for Flt1 and VEGF, but negative for Flk1. In the bronchiolar epithelium, ciliated cells showed weak immunoreactivity for Flt1, while Clara cells stained for Flk1 and for VEGF. Additional immunoperoxidase staining for Flk1 was observed to be associated with interstitial mast cells (Fig. 1e). Immunostaining was completely abolished by preabsorption of the antibodies with the respective control peptide (Fig. 1g).

The immunohistochemical findings were confirmed by means of double immunofluorescence labelling. Flt1 antigen was seen in ciliated bronchiolar epithelium, but not in Clara cells (Fig. 2a, b), and in alveolar type 2 pneumocytes (Fig. 2c, d), as evidenced by colocalization with established cell type-specific markers [24]. Flt1 immunoreactivity exhibited a cytoplasmic, finely granular distribution. This was supported by immunoelectron microscopy which revealed that Flt1 labelling was most prominently localised to multivesicular bodies of type 2 pneumocytes, in particular to internal vesicle membranes (Fig. 2e, f). As regards Flk1, double immunofluorescence labelling revealed strong colocalization of Flk1 with bronchiolar epithelial cells which stained intensely for SP-D indicating that Flk1 was present in Clara cells (Fig. 3a, b). In contrast, alveolar type 2 pneumocytes labelled with SP-D were devoid of Flk1 staining. While double labelling experiments using α-SM actin or MPA demonstrated the lack of any colocalization with Flk1 or Flt1 (not shown), double labelling for anti-mast cell tryptase and Flk1-antigen confirmed the presence of Flk1 in mast cells (Fig. 3e, f). Unfortunately, the antibody against Flk1 failed to work in immunoelectron microscopy of Lowicryl-embedded specimens.

By means of in situ hybridisation, the mRNAs of both Flt1 and Flk1 were detected in bronchiolar Clara cells and type 2 pneumocytes (Fig. 4). Thus, the distribution patterns of the transcripts differed slightly from the corresponding antigen distribution patterns.

Intratracheal application of bleomycin resulted in the characteristic histological alterations: fibrotic lesions associated with hyperplasia of type 2 pneumocytes, accumulation of alveolar macrophages, and collagen deposition [18, 25]. Early fibrotic lesions were seen predominantly at subpleural sites and were associated with complete loss of alveolar structure and moderate infiltration as well as with intra-alveolar clusters of foamy alveolar macrophages [28]. At later time points, formation of fibrosis was also seen at peribronchial sites.

In lungs of rats sacrificed at different time points (5–42 days) after intratracheal administration of bleomycin, there were no changes in the immunohistochemical

Table 1 Differential immunohistochemical distribution patterns of VEGF receptors Flt1 and Flk1 in normal and fibrotic rat lungs studied at different time points after intratracheal instillation of bleomycin (- unstained; (+) weak to moderate staining (of single cells), ++ intense staining, ⇑ increase in cell counts, ⇓ decrease in cell counts)

Experimental group (no. of animals)	Control (n=2)			5 days (n=3)			24 days (n=5)			28 days (n=4)			35 days (n=4)			42 days (n=5)		
Antigen	VEGF	Flt1	Flk1	VEGF	Flt1	Flk1	VEGF	Flt1	Flk1	VEGF	Flt1	Flk1	VEGF	Flt1	Flk1	VEGF	Flt1	Flk1
Bronchiolar epithelium																		
Ciliated cells	–	+	–	–	+	–	–	+	–	–	+	–	–	+	–	–	+	–
Clara cells	++	–	++	++	–	⇓	++	–	⇓	++	–	⇓	++	–	⇓	++	–	⇑
Alveolar epithelium																		
Type-1 pneumocytes	(–)	–	–	(–)	–	–	(–)	–	–	(–)	–	–	(–)	–	–	(–)	–	–
Type–2 pneumocytes	++	++	–	++⇑	++⇑	–	++⇑	++⇑	(+)	++⇑	++⇑	(+)	++⇑	++⇑	(+)	++⇑	++⇑	(+)
Alveolar macrophages	(+)	+	–	+⇑	+⇑	–	+⇑	+⇑	(+)	+⇑	+⇑	(+)	+⇑	+⇑	(+)	+⇑	+⇑	(+)
Myofibroblasts	(+)	–	–	(+)	–	–	++⇑	–	–	++⇑	–	–	++⇑	–	–	++⇑	–	–
Mast cells	–	–	++	–	–	++	–	–	++⇑	–	–	++⇑	–	–	++⇑	–	–	++⇑
Capillary endothelium	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–

Table 2 Results of double immunofluorescence labelling for Flk1 and SP–D in bronchiolar Clara cells of normal and bleomycin–treated rat lungs (*p.t.* after treatment)

Experimental groups	Total number of cell profiles	Flk1$^+$/SP-D$^+$ cell profiles[a] (%)[b]	Flk1$^-$/SP-D$^+$ cell profiles (%)[b]
Control	617	55±7	45±5
5 days p.t.	347	33±2	67±2
24 days p.t.	395	39±7	61±6
42 days p.t.	322	24±12	76±12

[a] *Flk1$^+$/SP–D$^-$* type of staining was seen in less than 1% of the cell profiles examined
[b] Means ±SEM of 2 animals per group

distribution pattern of Flt1, with the exception that fibrotic lungs were characterised by proliferation of type 2 pneumocytes and alveolar macrophages, which were positive for Flt1 (Table 1). In turn, the immunohistochemical distribution pattern of Flk1 changed early with the onset of pulmonary fibrosis and in association with alterations of the pattern of VEGF immunoreactivity (Table 1). As early as 5 days after bleomycin treatment, the fraction of Flk1-positive bronchiolar Clara cells had decreased from 55% to 33% of the total population of SP-D-staining Clara cells. This loss of expression of Flk1-antigen in Clara cells progressed with time after bleomycin treatment (Table 2). At 24 days after bleomycin treatment, when signs of interstitial fibrosis had developed, immunostaining for Flk1 was no longer restricted to bronchiolar Clara cells, but exhibited weak cytoplasmic immunoreactivity in type 2 pneumocytes and alveolar macrophages (Fig. 1h).

One of the most prominent features accompanying the development of fibrosis was the close association of Flk1-immunoreactive mast cells and VEGF-positive cells in fibrotic lesions (Fig. 1i, k). Notably, fibrotic lesions were characterised by the presence of α-SM actin-stained myofibroblasts, which by double immunofluorescence microscopy were shown to exhibit VEGF antigen [12]. While few mast cells were present in normal rat lungs and in lungs obtained 5 days after treatment with bleomycin and those observed were restricted to the peribronchiolar, perivascular and pleural interstitium, they accumulated progressively in developing fibrotic lesions, until they had densely populated areas of prominent fibrosis (Fig. 5).

Discussion

Pulmonary fibrosis is characterised by a profound remodelling of the lung parenchyma. This complex process involves the proliferation and migration of resident cells as well as the immigration of inflammatory and immune effector cells [8, 50, 55]. All these different cell types interact by means of a variety of cytokines some of which have been reported to be involved in fibrogenesis [60]. Recently, vascular endothelial growth factor

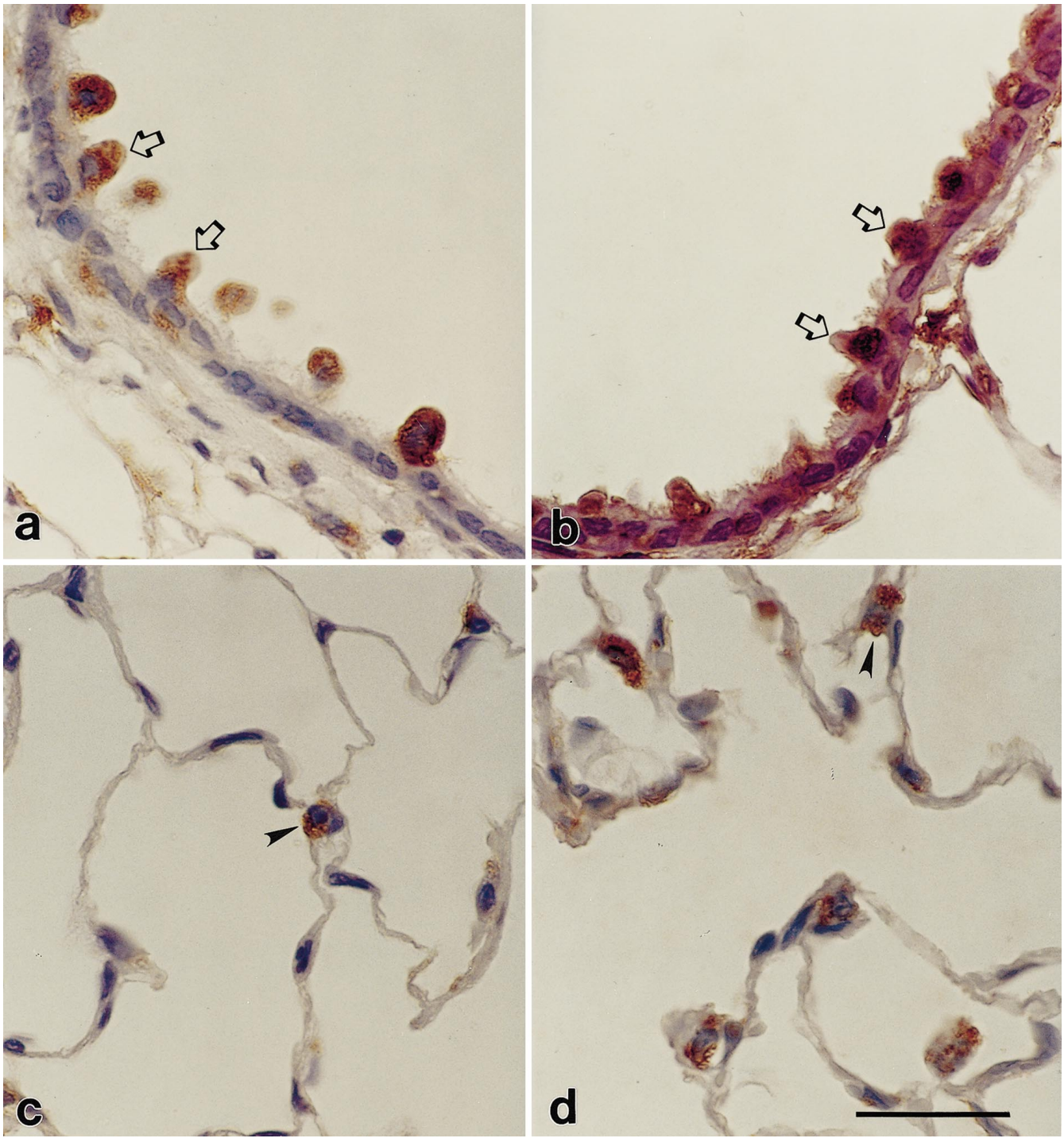

Fig. 4 In situ hybridisation revealed the presence of transcripts of **(a, c)** Flt1 and **b, d** Flk1 in bronchiolar Clara cells (*open arrows*) and alveolar type 2 pneumocytes (*arrowheads*) in control lungs. *Scale bar* 20 μm

(VEGF) has been suggested as one of the actors in pulmonary fibrosis [12].

The functions of VEGF and its receptors Flt1 and Flk1 are related mainly to angiogenesis in embryonic development and in tumor formation [10, 40, 47]. VEGF protein and mRNA are present in alveolar type 2 pneumocytes and Clara cells of normal adult lungs [7, 12, 35, 39, 48, 52, 59], but the functional significance of epithelial VEGF in the adult lung remains obscure. It has been suggested that the abundant presence of VEGF in the alveolar epithelium might reflect some paracrine action on the microvascular endothelium [7]. In the developing human lung, VEGF appeared to be synthesised by distal airway epithelial cells and translocated to the subjacent basement membrane, which might be important in directing pulmonary capillary development [1]. Similarly,

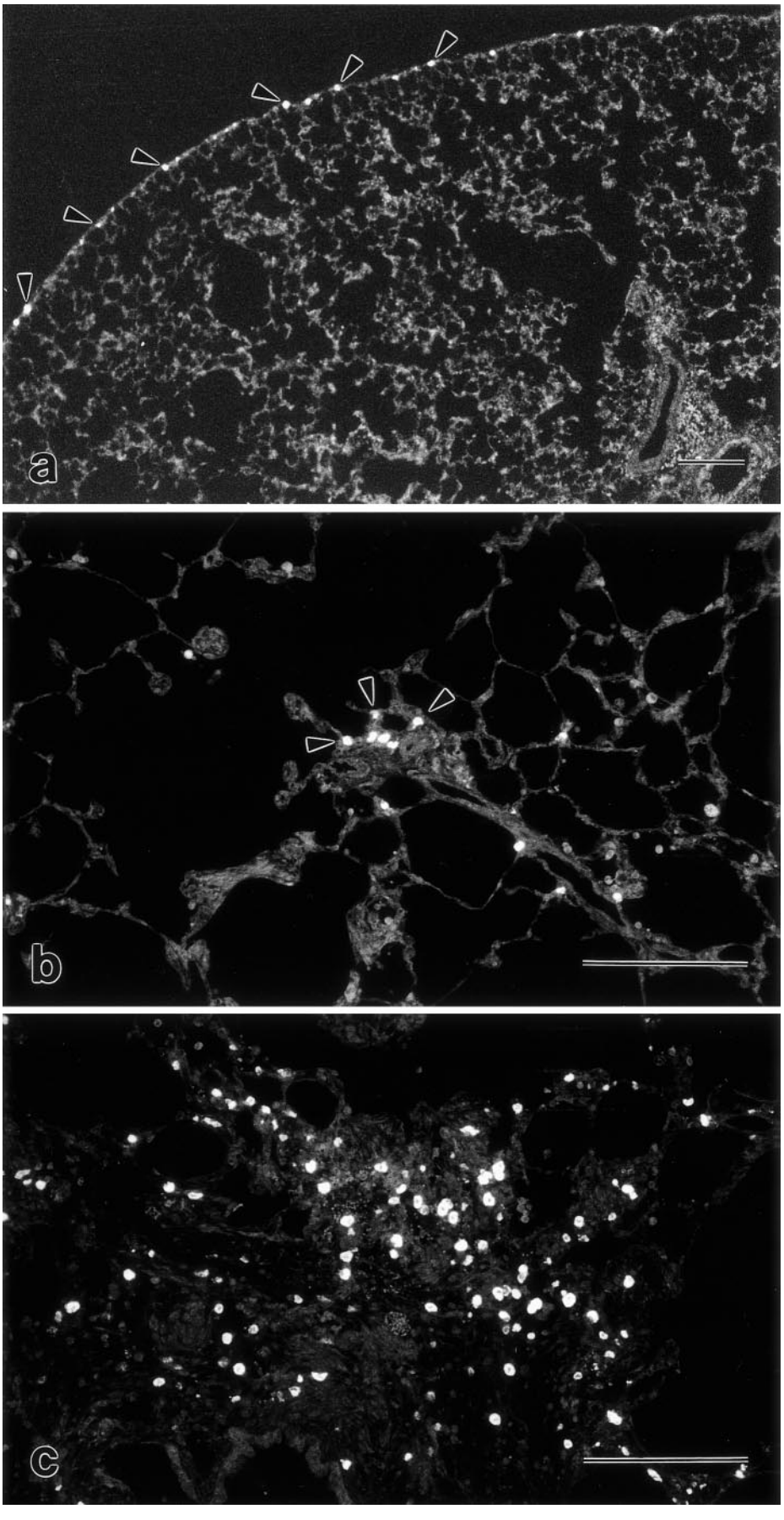

Fig. 5 Immunofluorescence staining for mast cell tryptase. **a** Five days after treatment with bleomycin mast cells (*arrowheads*) were almost exclusively seen to reside in the pleura. **b** They progressively accumulated at sites of early fibrotic lesions observed 24 days after treatment, and **c** had densely populated areas of advanced pulmonary fibrosis at 42 days after bleomycin treatment. *Scale bars* 200 µm.

the presence of VEGF in vascular smooth muscle has been proposed to be involved in the maintenance of normal vascular function [47]. However, direct evidence to support these hypotheses is still lacking. Our immunohistochemical findings of predominant localisation of both VEGF and its receptors Flt1 and Flk1 in the pulmonary epithelium suggest a role for this cytokine outside the context of vascular function in the adult lung. Using double immunofluorescence microscopy and a number of well-established cell markers [23, 24], VEGF immunoreactivity was detected in alveolar type 2 pneumocytes and in Clara cells of the bronchial epithelium [12].

Flt1 antigen and mRNA were shown to be present in type 2 pneumocytes, and Flk1 antigen and mRNA were detected in bronchial Clara cells. However, while Flt1-antigen was seen on ciliated bronchial epithelium, Flt1-mRNA was present in Clara cells. Although we cannot resolve this discrepancy between immunohistochemistry and in situ hybridisation conclusively, one may assume that Clara cells secrete a soluble form of Flt1 (sFlt1), which might be distributed over the bronchial epithelium. The existence of sFlt1 has recently been demonstrated, and sFlt1 was shown to be able to bind VEGF, to form heterodimers with Flk1, and to be a presumptive paracrine inhibitor of tumor angiogenesis [14, 29]. As regards Flk1, in situ hybridisation revealed the presence of Flk1 mRNA not only in Clara cells but also in type 2 pneumocytes of control lungs, while in this cell type Flk1 antigen could only be seen in fibrotic lungs. This may indicate that the mRNA of Flk1 is expressed but not translated into protein in type 2 cells of normal rat lung, while protein synthesis may be induced during fibrosis. An analogous mechanism of regulation has been revealed for interleukin 1 [44].

While VEGF-binding sites were initially reported to be restricted to endothelial cells [21], recent studies have yielded evidence of a much more widespread occurrence of the receptors for VEGF in nonendothelial cells. Flt1 is found in human syncytiotrophoblast, Hofbauer cells, and maternal decidual cells [4] and also in activated alveolar macrophages, epitheloid cells, and multinuclear giant cells of pulmonary sarcoid granulomas [51]. Flk1 is also present in neural progenitor cells of the mouse retina [57] and in rat pancreatic duct cells [45], and both Flt1 and Flk1 are found in uterine but not in colonic smooth muscle cells [6] and in Leydig, Sertoli and perivascular cells of human testis in Man [11] and in the mouse [30]. On the basis of the differential distribution of VEGF and its receptors in the pulmonary epithelium, it is tempting to propose that in the adult rat lung VEGF may act as an autocrine factor on alveolar type 2 pneumocytes (via Flt1) and on Clara cells (via Flk1), while feedback loops may exist involving a Clara cell-derived sFlt1. However, it must be taken into account that Flt1 is not only a receptor for VEGF but also for the related growth factor PlGF [40]. Some evidence supporting the notion that VEGF may exert some biological action on the pulmonary epithelium is indicated by the recent finding of an inhibition of the differentiation of type 2 into type 1 pneumocytes in transgenic SP-C-VEGF mice which showed an elevated level of VEGF-mRNA along malformed acinar tubules and buds consisting of a columnar type 2-like epithelium [59]. However, there is no direct evidence for any of the relationships suggested in this or in other studies [7, 47].

Based upon the observation that fibrotic lesions were characterised by an increase in VEGF-immunoreactive cells including α-SM actin-containing myofibroblasts and type 2 pneumocytes, it has been suggested that VEGF is involved in bleomycin-induced lung fibrogenesis [12]. This hypothesis is supported by the findings presented in this study. The most intriguing effect observed after bleomycin treatment was the accumulation of Flk1-positive mast cells in fibrosing regions of bleomycin-treated rat lungs. Cell type specificity was confirmed by double immunofluorescence labelling with a monoclonal anti-mast cell tryptase antibody, and preabsorption controls were negative. While in normal rat lung, only a few mast cells were seen in the peribronchovascular and subpleural interstitium, mast cells appeared in the parenchyma in early fibrotic lesions and densely populated regions with advanced fibrotic alterations. These observations are in line with reports of a considerable increase in the number of mast cells during bleomycin-induced [15, 18] and radiation-induced fibrogenesis [53]. Moreover, mast cell counts were higher in patients with fibrotic lung disorders than in control lungs [42]. Recently, VEGF has been shown to act as a chemoattractant for mast cells [16]. In addition, in transgenic mice, chronic VEGF overexpression by epidermal keratinocytes was associated with an accumulation of mast cells in the upper dermis [9]. As we have shown in this study, the accumulation of mast cells in fibrotic lung lesions may be due to chemotaxis mediated by the interaction of VEGF generated by myofibroblasts and/or type 2 pneumocytes, with its receptor Flk1, present on the mast cell membrane. There is a body of evidence that mast cells release mediators such as basic fibroblast growth factor and mast cell tryptase that stimulate fibroblast proliferation and collagen synthesis [17, 19, 20, 46]. As a consequence, the release of these fibroblast-stimulating factors may result in a vicious circle, with further proliferation of VEGF-generating myofibroblasts attracting additional mast cells, and so forth. Notably, mast cell counts obtained from lung biopsy specimens of patients with fibrotic lung disorders were reported to correlate significantly with the degree of fibrosis [42].

In conclusion, our immunohistochemical findings support the notion that VEGF together with its receptor Flk1 is involved in the pathogenesis of bleomycin-induced pulmonary fibrosis. VEGF/Flk1 may represent a molecular link between the proliferation of myofibroblasts, the accumulation of mast cells, and the burst of fibrosis at sites of initial lesions.

Acknowledgements The authors are greatly indebted to Professor Dr. K.-W. Wenzel (Institute of Physiological Chemistry, TU Dresden, Germany) for his support. The expert technical assistance of I. Peterson, R. Petzold, and S. Langer in immunohistochemical and immunofluorescence staining, of B. Kettner, B. Rudolph, and

C. Tränkner in immunoelectron microscopy, of A. Lengnick in in situ hybridisation, and of H. Seidel for photographic reproduction is acknowledged with thanks. We gratefully acknowledge the cooperation of Dr. G. Breier (Bad Nauheim, Germany) and Dr. B.I. Terman (Pearl River, NY, USA) who kindly provided the plasmids used to generate the probes for in situ hybridisation. The study was supported by the Bundesministerium für Bildung und Forschung (01ZZ5904).

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