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Cellular distribution of c-Jun and c-Fos in rat lung before and after bleomycin induced injury

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Abstract C-Jun and c-Fos transcription factors have been associated with enhanced cellular proliferation. We studied their cellular distribution in normal and fibrotic rat lung. Pulmonary fibrosis was induced by intratracheal administration of bleomycin. In normal rat lung, c-Jun and c-Fos are present in alveolar macrophages and type II pneumocytes, in the bronchiolar epithelium and in smooth muscle cells of bronchioli and blood vessels. Subcellular fractionation of proteins revealed a predominant presence of both c-Jun and c-Fos in the heavy membrane fraction containing mitochondria and secretory granules. This was confirmed by immunoelectron microscopy, which also revealed a different localization of c-Jun and c-Fos in different cell types. Whereas in type II pneumocytes and in macrophages cytoplasmic c-Jun and c-Fos is associated with mitochondria, in Clara cells of the bronchial epithelium only secretory granules contain c-Jun and c-Fos. In addition, c-Jun is strongly present in the nuclear fraction. In the fibrotic rat lung c-Jun and c-Fos are located in the same cell types as in control lungs. In addition, fibroblasts contain c-Jun and c-Fos in areas of proliferation whereas in areas of complete fibrosis there is only a very weak expression of c-Jun and c-Fos.

Key words Transcription factor AP-1 · Pulmonary fibrosis · Bleomycin · Mitochondria · Proliferation

Introduction

C-Jun and c-Fos, which belong to the AP-1/b-ZIP family of transcription factors, have been associated with inflammatory and proliferative processes [4, 15, 23, 29, 32]. C-Jun is able to activate gene transcription by forming homodimers or heterodimers with other Jun/Fos proteins [26].

Pulmonary fibrosis is a proliferative disease with potentially fatal prognosis (for review see [11]). It is characterized by the proliferation of several cell types, including fibroblasts and type II pneumocytes, together with increased accumulation of connective tissue components in the interstitium. Several growth factors and cytokines known to be involved in fibrogenesis are transcriptionally regulated by AP-1, among them TNFα [17, 21, 22], PDGF [2] and TGFβ [6]. Promotors of metalloproteinases, such as collagenase [12, 14], integrins such as alpha-2 integrin, as part of the collagen/laminin receptor [13] and also the receptor for hyaluronic acid CD 44 [3] contain AP-1 binding sites.

One of the clinically important causative agents in pulmonary fibrosis is the anticancer drug bleomycin [1]. Lung toxicity occurs via formation of oxygen free radicals, because the activity of the bleomycin-inactivating enzyme, bleomycin hydrolase, is very low in the lung [20].

We studied the cellular distribution of c-Jun and c-Fos to find what cell types might potentially be involved in gene regulation by c-Jun and c-Fos during bleomycin-induced pulmonary fibrosis.

Materials and methods

Female Wistar rats (n = 29) each weighing about 200 g received endotracheally a single dose of 7 U bleomycin sulfate per kg body weight dissolved in 0.25 ml 0.9% NaCl. Controls (n = 3) were given saline alone. At 4 (n = 3), 8 (n = 4) and 12 h (n = 3), 1 (n = 2) and 5 (n = 5) days, 3 (n = 3), 4 (n = 2), 5 (n = 3) and 6 (n = 4) weeks after bleomycin administration the animals were killed, and after perfusion of the lungs the lower part of the right lobe was taken for fixation in 4% buffered formaldehyde and subsequent paraffin embedding. The care and use of the animals reported on in this study were approved by the Animal Care Unit of the Technical University of Dresden.

For immunohistochemistry dewaxed sections were either pretreated with pronase (1 mg/ml, 10 min at 37°C) or microwaved

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Table 1 Primary antibodies used, pretreatment (*MW* microwave) and dilutions for immunoperoxidase staining (*I-POX*) and immunofluorescence staining (*I-FLU*); indication of the presence of nuclear (*N*) and cytoplasmic (*C*) staining within the cells by those antibodies (*pR* polyclonal rabbit, *mM* monoclonal mouse)

Antibody (Ab)	Kind of Ab	Company	Pretreatment	Dilution I-POX	Dilution I-FLU	Subcellular staining
c-Jun/AP-1 (D) X	pR	Santa Cruz biotech[a]	MW	1:50	1:25	C>N
c-Jun/AP-1 (N) X	pR	Santa Cruz biotech[a]	MW	1:50		C>N
c-Jun/AP-1 (KM-1)	mM	Santa Cruz biotech[a]	MW	1:10		N
c-Jun/AP-1 (Ab-1)	pR	Oncogene Science[b]	MW	not diluted		C>N
c-Jun/AP-1 (Ab-2)	pR	Oncogene Science[b]	Pronase	1:50	1:25	C>N
c-Jun (Ab-4)	pR	Oncogene Science[b]	MW	1:100		C>N
c-Jun (3-TL)	mM	Transduction Lab.[c]	MW	1:3 (ABC)		N>C
c-Fos (4) X	pR	Santa Cruz Biotech[a]	MW	1:30		C>N
c-Fos (Ab-2)	pR	Oncogene Science[b]	MW	1:10		C>N
c-Fos (Ab-4)	pR	Oncogene Science[b]	Pronase	1:10	1:5	C>N

[a] Heidelberg, Germany
[b] Dianova, Hamburg, Germany
[c] Affiniti, Exeter, UK

Table 2 Cellular distribution[a] of c-Jun and c-Fos in normal and fibrotic rat lung

	Alveolar epithelial cells		Bronchial epithelial cells		Alveolar macrophages	Mast cells	Fibroblasts	Smooth muscle cells		Endothelial cells
	type I	type II	ciliated	Clara cells				Arterial	Bronchial	
Normal lung										
c-Jun	0	3	2	3	3	n.p.	0	2	2	0
c-Fos	0	2	2	3	3	n.p.	0	2	2	0
Fibrotic lung										
c-Jun	0	3	2	3	3	1	2	2	2	0
c-Fos	0	2	2	3	2	1	2	2	2	0

[a] Semiquantitative scoring of labelling intensity: *0* none (labelling does not exceed background levels); *1* slight (diffuse, low-level labelling); *2* moderate (distinct labelling, exceeds most other immunostained areas); *3* heavy (dense labelling, conspicuous against all immunostained areas); *n.p.* cells not present in normal lung

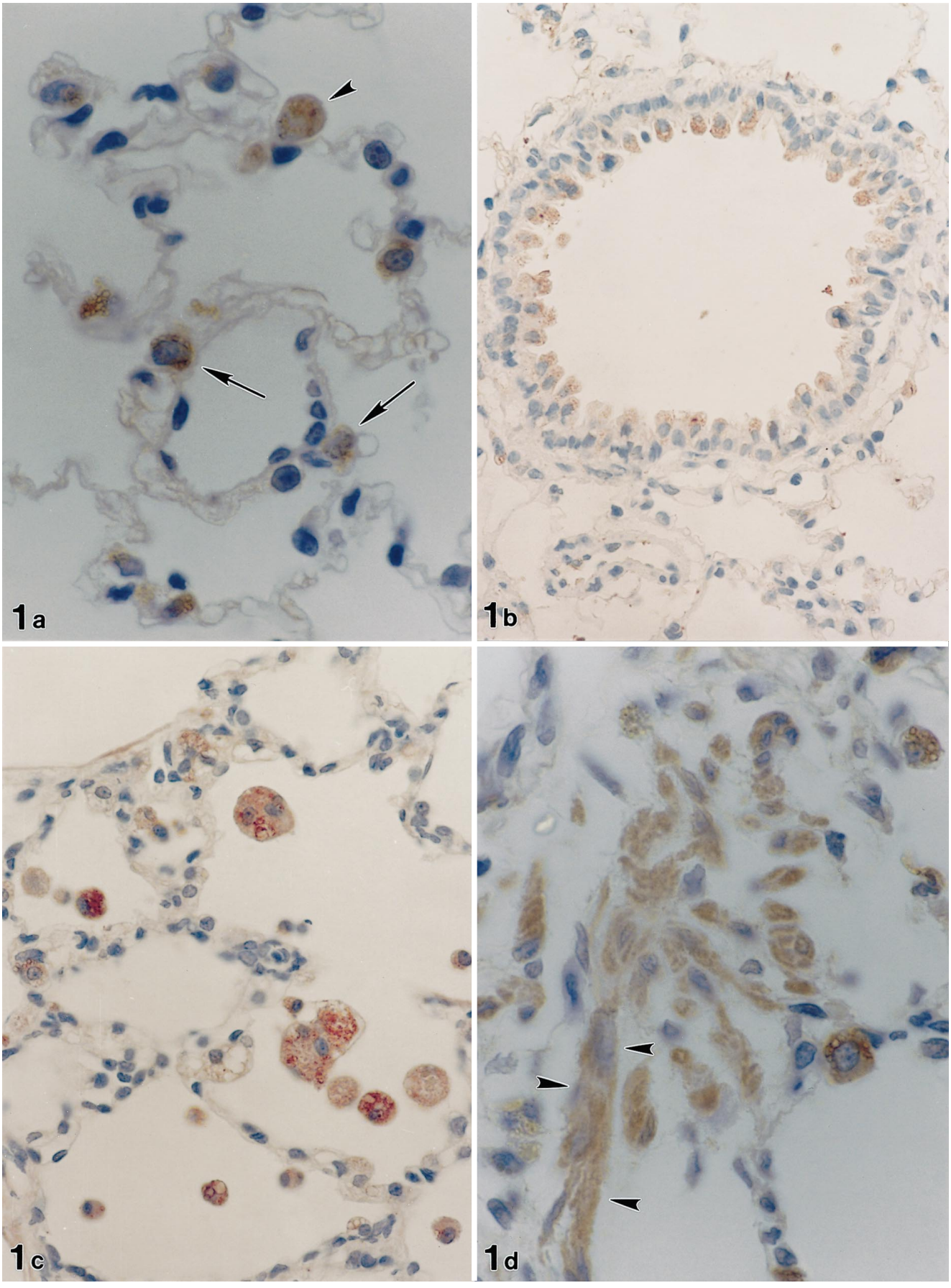
(MW) for 3×5 min at 750 W in 10 mM citric acid, pH 6.0 [18] and, after blocking with normal serum, incubated with the primary antibody for 1 h at 37°C as indicated in Table 1. After 10 min washing in PBS, sections were incubated with the secondary antibody (HRP conjugted goat anti-rabbit antibody, dilution 1:400) for 1 h at RT. Negative controls included omission of the primary antibody and its replacement by PBS or normal rabbit serum. The conjugates were visualized with diaminobenzidine. Nuclei were counterstained with haematoxylin.

For immunofluorescent double labelling the pretreatment of the sections was the same as above. Concentrations for incubation with the primary antibodies to c-Jun and c-Fos are shown in Table 1. For verification of the cell types, slides were additionally incubated with a monoclonal antibody to surfactant protein D (SP-D) [19] (not diluted), a polyclonal antibody to smooth muscle actin (Immunotech, Hamburg, Germany, not diluted) and a monoclonal antibody to mast cell tryptase (clone AA1, Dako, Hamburg, Germany, dilution 1:3). Sections were incubated with the primary antibodies for 45 min at RT. After 10 min washing with PBS, slides were incubated with Texas red conjugated anti-mouse antibody (Dianova, Hamburg, Germany, dilution 1:80) or dichlorotriazinyl aminofluorescein (DTAF)-conjugated anti rabbit antibody (Dianova, Hamburg, Germany, dilution 1:40) for 30 min at RT. For negative controls, primary antibodies were omitted or replaced by non-immune rabbit or mouse IgG. Slides were finally mounted in PBS–glycerol (1:9).

Pieces of rat lung 1–2 mm^2 in area were fixed for immunoelectron microscopy in 4% paraformaldehyde (in 0.1 M cacodylate buffer), frozen in liquid nitrogen, cryosubstituted and embedded in Lowicryl HM20. Ultrathin sections were mounted on colloidin-coated nickel grids. Grids were incubated with 10% normal goat serum in Tris-buffered saline (TBS), pH 8.2, for 45 min at RT and then with the antibody to c-Jun/AP-1(D)X (Santa Cruz Biotech) at a dilution of 1:5 or the antibody to c-Fos(4)X (Santa Cruz Biotech) at a dilution of 1:3 for 1 h at RT. After washing with buffer (TBS containing 0.2% BSA) sections were incubated with 20 nm gold-conjugated goat anti rabbit antibody (1:50 in TBS, pH 8.2) for 1 h at RT. After washing with buffer, the sections were stained with 2.5% uranylacetate in 50% ethanol and 1% lead citrate for 2 min each.

To carry out subcellular fractionation and Western blotting, normal rat lungs were used to collect proteins from subcellular fractions as described by Hockenbery et al. [17], using the buffers described by Schreiber et al. [28] with modifications. Frozen pieces of tissue about 5 mm in diameter were homogenized for 1 min

Fig. 1a–d Immunoperoxidase staining of paraffin sections for c-Jun and c-Fos in normal and fibrotic rat lung. **a** c-Jun in normal lung using c-Jun/Ap-1(Ab-2) antibody: type II cells (*arrows*) and an adherent alveolar macrophage (*arrowhead*). ×1000. **b** c-Fos in normal lung using c-Fos (Ab-4) antibody: positivity of Clara cells and ciliated bronchial epithelial cells. ×400. **c, d** c-Jun in bleomycin-treated lung using c-Jun/Ap-1(Ab-2) antibody. ×1000. **c** Thickenting of alveolar walls and accumulation of enlarged alveolar macrophages. **d** Proliferation of fibroblasts (*arrowheads*)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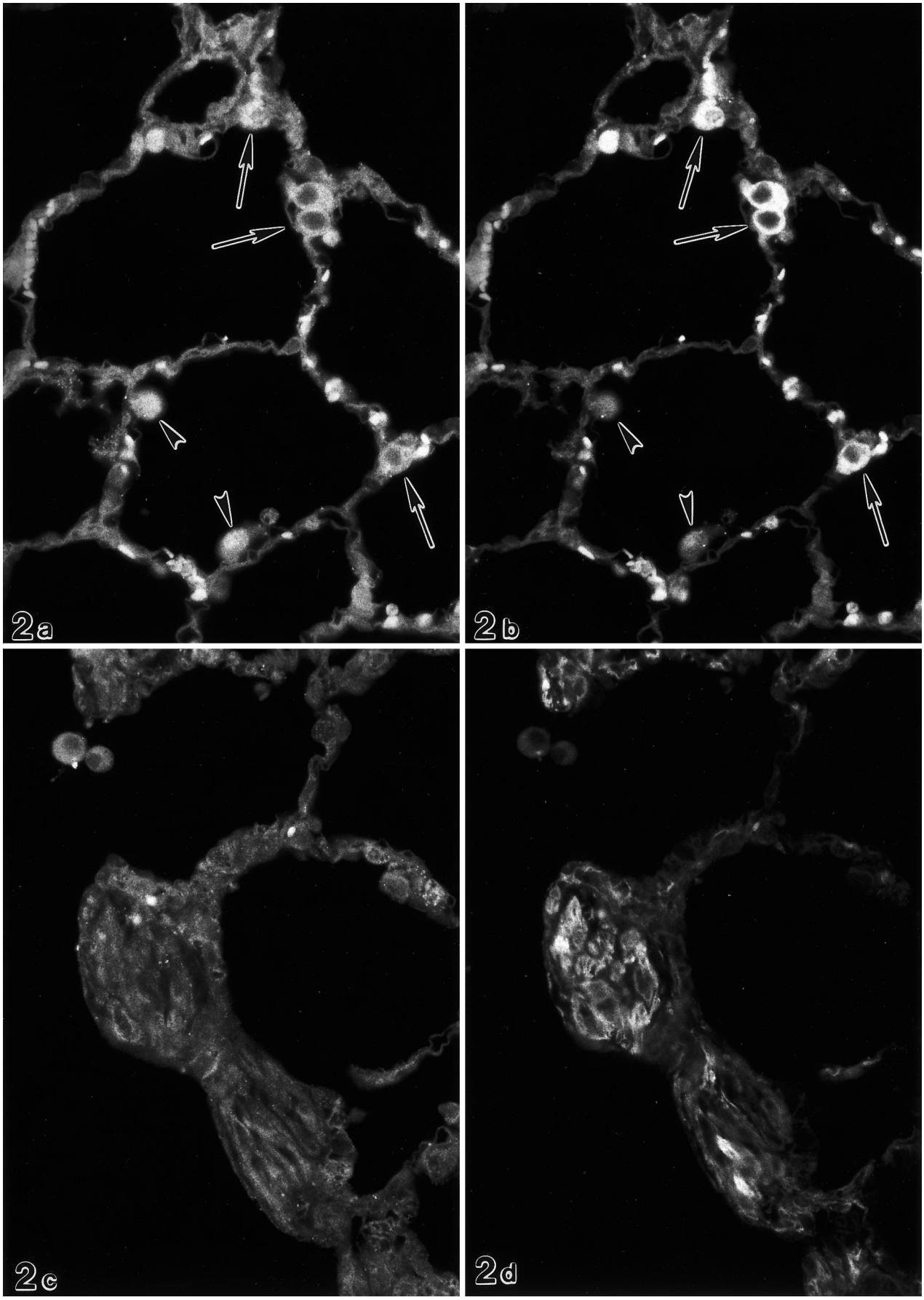
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in 2 ml hypotonic buffer A (10 mM Hepes, pH 7.9, 10 mM KCl, 0.1 mM EGTA, 0.1 mM EDTA, 1 mM DTT, 0.5 mM PMSF) using an ultraturrax (IKA Labortechnik, Staufen, Germany). After a 10 min incubation nuclei were pelleted by centrifugation at 800 *g* for 10 min. Pelleted nuclei were resuspended in 100 µl buffer B (20 mM Hepes, pH 7.9, 400 mM NaCl, 1 mM EGTA, 1 mM EDTA, 1% Triton X-100, 0.5% sodium deoxycholate, 0.5% NP40, 1 mM DTT, 0.5 mM PMSF). The supernatant was centrifuged at 10,000 *g* for 10 min to collect the "heavy membrane" fraction (mitochondria, lysosomes, secretory granules). The pellet was suspended in 80 µl of buffer B. This supernatant was centrifuged at 100,000 *g* to collect the "light membrane" fraction containing lower molecular weight organelles (Golgi membranes etc.). The pellet was suspended in 60 µl of buffer B. The supernatant containing free cytoplasmic proteins was adjusted to a final concentration of 100 mM NaCl, 0.25% Triton X-100, 0.13% sodium deoxycholate, 0.13% NP-40 and concentrated to 500 µl using a speedvac. Then 50 µg proteins from each fraction were electrophoresed on a 10% polyacrylamide gel using standard methods [25], blotted on an ECL nitrocellulose membrane and developed using the ECL-Kit from Amersham (Braunschweig, Germany) according to the manufacturer's instructions.

Results

In the normal lung, c-Jun and c-Fos were located in the cytoplasm of alveolar macrophages and type II pneumocytes (Table 2, Fig. 1). The immunostaining showed a granular or vesicular pattern, suggesting that organelles are stained (Fig. 1a). Double-label immunofluorescence using the monoclonal antibody against SP-D and the polyclonal antiserum to c-Jun or c-Fos confirmed the predominant staining of type II pneumocytes and alveolar macrophages (Fig. 2a, b). In the bronchiolar epithelium, Clara cells were strongly positive, but so also were ciliated bronchiolar epithelial cells (Fig. 1b). Smooth muscle cells of bronchioli and blood vessels were moderately stained. Specifity of the staining reactions of two characteristic antibodies [c-Jun(N)X and c-Fos(4)X] were confirmed by blocking experiments with specific and nonspecific peptides (data not shown).

Subcellular fractionation of whole-lung extracts using differential centrifugation and subsequent Western blotting revealed that both c-Jun and c-Fos are located in the heavy membrane fraction containing mitochondria, secretory granules and lysosomes (Fig. 4). The light membrane fraction (endoplasmatic reticulum, Golgi vesicles) contains only a small amount of c-Jun and c-Fos. In contrast to c-Fos, c-Jun is also present in the nuclei of the cells in large amounts. Free cytoplasmic c-Jun or c-Fos is only very rarely detectable. The nonspecific band around 81 kDa in the heavy membrane fraction of c-Fos might represent a complex with (an)other protein(s).

The staining of organelles was confirmed ultrastructurally, using immunoelectron microscopy (Table 3). In addition, different cell types showed a different localization of c-Jun and c-Fos. Mitochondria of type II pneumocytes and macrophages are stained with antibodies to both c-Jun and c-fos (Fig. 3a, b), whereas the mitochondria of Clara cells are not. In those cells, the (secretory) granules stain positive (Fig. 3c, d). The lysosomes of macrophages show very weak staining. The nuclei of all cell types are moderately stained for c-Jun and weakly for c-Fos.

Table 3 Ultrastructural localization of c-Jun and c-Fos in normal rat lung cells (semiquantitative scoring as in Table 2)

	Nuclei	Mitochondria	Secretory granules	Lysosomes
Type-II cells				
c-Jun	2	3	0	
c-Fos	1	2	0	
Clara cells				
c-Jun	2	0	3	
c-Fos	1	0	3	
Macrophages				
c-Jun	2	3		1
c-Fos	1	3		1

Most of the antibodies used stain cytoplasmic more than nuclear c-Jun and c-Fos (Table 1, last column). Only the antibodies c-Jun (KM-1) and c-Jun/AP-1(3-TL) show more nuclear than cytoplasmatic, or only nuclear staining of those proteins.

In comparison with control lungs there were no changes in the expression of either c-Jun or c-Fos 1 h to 1 week after bleomycin administration. There were no changes in cytoplasmic compared with nuclear staining.

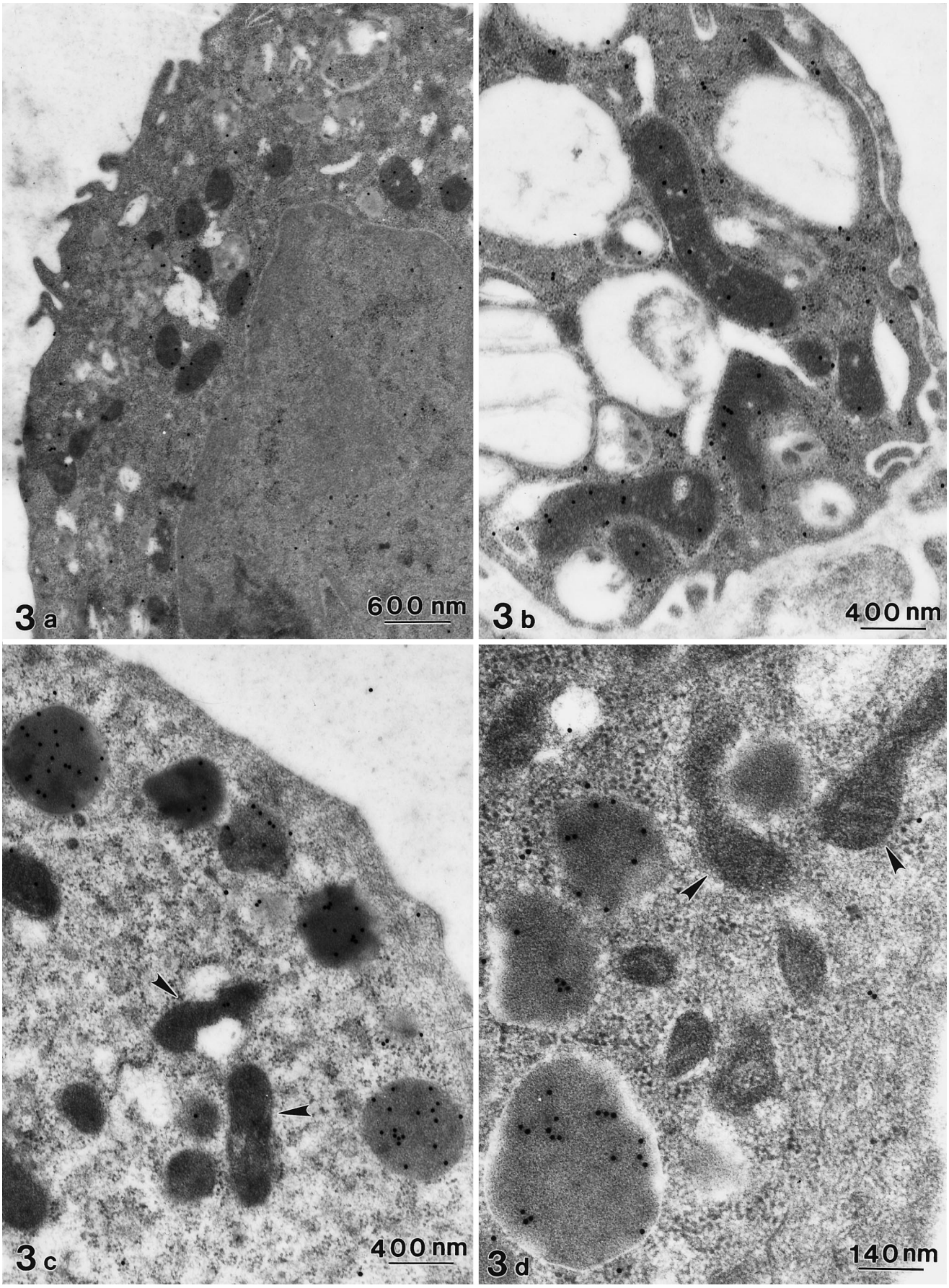
Pulmonary fibrosis is seen in lungs from 3 weeks until 6 weeks after bleomycin administration. Later stages (6 weeks after bleomycin application) show a higher percentage of fibrosis, but there is no difference in the quality of the staining pattern from 3 to 6 weeks after bleomycin application.

In the fibrotic lung, alveolar macrophages and type II cells are positive with no significant increase in staining, but they are larger than in controls and seem to contain large granules loaded with c-Jun (Fig. 1c) and c-Fos. The staining of the bronchiolar epithelium and the smooth muscle cells is not changed. Mast cells, which emerge in fibrotic areas, show only very weak staining of both c-Jun and c-Fos. In addition to control lungs, myofibroblasts in areas of incipient fibrosis and in the borders of larger areas of fibrosis are positive (Figs. 1d, 2c, d). Fibroblasts located in the centre of larger areas of fibrosis have a lower content of both c-Jun and c-Fos. There is no increase in nuclear staining in the cells of fibrotic lungs.

Discussion

We found that normal and pathologic pulmonary cells known to be actively involved in the process of fibrosis, particularly alveolar macrophages and type II pneumo-

Fig. 2 Paraffin sections of **a, b** normal and **c, d** fibrotic rat lung. ×400. Double immunofluorescence demonstration of c-Jun using **a** c-Jun/AP-1(D)X antibody and **b** SP-D in type-II pneumocytes (*arrows*) and in alveolar macrophages (*arrowheads*). **c, d** Simultaneous localization of **c** c-Fos using c-Fos (Ab-4) antibody and **d** smooth muscle actin in myofibroblasts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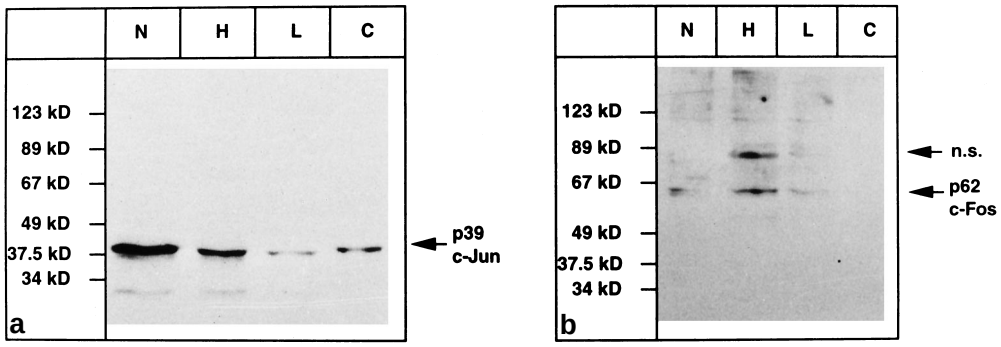

Fig. 4 Subcellular distribution of **a** c-Jun and **b** c-Fos in normal rat lung using mAb c-Jun/AP-1 (3-TL) and pAb c-Fos (4)X antibodies (*N* nuclear proteins, *H* heavy membrane proteins *L* light membrane proteins *C* cytoplasmic proteins *n.s.* nonspecific band)

cytes, contain high amounts of c-Jun and c-Fos. In addition, c-Jun and c-Fos are constitutively expressed in smooth muscle cells of bronchioli and blood vessels. In the fibrotic process, fibroblasts appear in large numbers in the alveolar walls and they, too contain c-Jun and c-Fos.

We found c-Jun and c-Fos both in the cytoplasm and in the nuclei of the cells detected by immunohistochemistry, subcellular fractionation followed by Western immunoblotting and immunoelectron microscopy. In the nucleus, transcription factors bind to their target sequences in order to regulate transcription. The cytoplasm is the location of transcription factors after their synthesis and prior to activation [8, 31]. However, it has not been shown in which subcellular compartment c-Jun and c-Fos reside before their translocation to the nucleus. One might expect the Golgi field to be a candidate of such a storage pool prior to activation. Surprisingly, we found c-Jun and c-Fos in the heavy membrane fraction containing mitochondria and other "heavy" organelles like lysosomes and secretory granules by means of subcellular fractionation followed by Western immunoblotting. This was confirmed by immunoelectron microscopy, which also revealed a different subcellular localization in different cell types. Whereas in type-II pneumocytes and alveolar macrophages c-Jun and c-Fos are associated with mitochondria, mitochondria of Clara cells are negative. Instead, c-Jun and c-Fos are located in the secretory granules of Clara cells. This difference in the subcellular localization of those immediate–early genes and stress-related transcripiton factors may indicate a different susceptibility of those cells towards various forms of cellular stress, such as bleomycin treatment. The fact that some of the antibodies used only weakly detect nuclear c-Jun and c-Fos may be explained by masking of epitopes owing to dimerization or DNA binding of the antigens. Since we did not detect c-Jun or c-Fos in other cytoplasmic compartments than those mentioned, we assume that mitochondria are the place from where c-Jun and c-Fos are translocated into the nucleus of type II pneumocytes and alveolar macrophages. There are no data available to indicate whether those transcription factors might also regulate transcription of mitochondrial DNA.

The molecular mechanisms involved in lung fibrogenesis are poorly understood and still largely unexplored. Althought different transcription factors can be activated by agents involved in lung injury [5, 7, 9, 16, 22, 27], no clear picture yet exists of the biological role they might have in the development of pulmonary fibrosis. C-Jun and c-Fos genes encode transcription factors that belong to the AP-1/bZIP family. As early response genes they are rapidly induced following growth factor stimulation [10, 23, 24, 30, 33] and in proliferative processes [4]. In our model, however, there are no changes in the distribution of c-Jun and c-Fos in the early stages of bleomycin injury. This does not necessarily represent unchanged functional activity, but it might indicate that no major changes occur over quite long periods (days or weeks). In fibrotic lungs, myofibroblasts in areas of active fibrosis contain c-Jun and c-Fos. Other cells show no changes in staining intensity. Type II pneumocytes and alveolar macrophages become larger and seem to contain more c-Jun and c-Fos per cell, but there is no increase in staining intensity, especially over the nuclei. The absence of a significant increase of staining intensity of c-Jun and c-Fos in most of the lung cells leads us to suggest that (1) a different regulatory pathway is involved in control of gene expression at manifest stages of lung fibrosis and/or (2) the activation of c-Fos and c-Jun transcription factors is inhibited by a so far unknown mechanism. It has to be taken into consideration that the generation of oxygen free radicals by bleomycin might lead to suppression of AP-1 activity [22]. This, however cannot be a sufficient explanation for the long-lasting effects. Alternatively, since the process of developing pulmonary fibrosis is characterized by a relatively slow proliferation over weeks and months, a permanent slow activation state slightly higher than the level in normal lungs cannot be detected by immunohistochemical methods.

Further experiments are needed to evaluate the expression of these and other transcription factors at early inflammatory stages of lung injury and in the course of developing fibrosis.

Fig. 3a–d Ultrastructural localization of c-Jun in control lungs. **a** Mitochondria of an alveolar macrophage staining positive with a c-Jun antibody. ×19800. **b** Type-II cell with mitochondria positive for c-Jun staining. ×28800. **c, d** Clara cell with secretory granules containing **c** c-Jun, nonstained mitochondria marked by *arrowheads* and **d** c-Fos. **c** ×28,800, **d** ×78,720

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