Int. J. Exp. Path. (1996), **77,** 155–161

Heparin attenuates bleomycin but not silica-induced pulmonary fibrosis in mice: possible relationship with involvement of myofibroblasts in bleomycin, and fibroblasts in silica-induced fibrosis

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Received for publication 18 January 1996
Accepted for publication 26 April 1996

Summary. Pulmonary fibrosis was elicited in mice or rats by the intra-tracheal instillation of bleomycin or silica. Daily injections of heparin significantly reduced the collagen deposition in bleomycin, but not in silica, injected mice, as evaluated by the lung hydroxyproline content on day 15 after instillation. Heparin also reduced the bleomycin-induced morbidity and mortality. Study of the broncho-alveolar lavage fluid (BAL) detected no significant change in the number of leucocytes or the amount of protein in heparin treated mice. Histologies of bleomycin instilled mice suggested that heparin did reduce the alveolar remodelling but not the alveolitis, evidenced by leucocytic infiltration. As detected by electron microscopy (EM), bleomycin increased the number of leucocytes and platelets within the alveolar capillaries but this was not significantly reduced by heparin. The phenotype of the interstitial cell involved in these two types of pulmonary fibrosis was investigated by immunohistochemistry and EM. While in bleomycin injected animals the interstitial cells had the phenotype of an actin (α-actin in the rat) and lipid containing interstitial cell, with a poorly developed RE, in silica injected animals in contrast, the interstitial cells were without cytoplasmic actin or lipid but with a markedly developed endoplasmic reticulum (ER). Thus bleomycin and silica induced the growth of two different types of interstitial cells, the myofibroblast and the regular fibroblast, which might be a reason why heparin selectively inhibits bleomycin but not silica-induced fibrosis.

Heparin is a negatively charged glycosaminoglycan produced by mast cells. It inhibits thrombin and is widely used as an anticoagulant. In addition, heparin has been reported to exert many other effects by interfering with the activity of cytokines (Yang & Yang 1995), proteases (Hagege *et al.* 1994) and integrin (Nelson *et al.* 1993).

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In vivo, heparin has been found to exert anti fibrogenic properties in glomerular disease (Tang & Wilson 1992), myocardial inflammation and fibrosis (Frizelle *et al.* 1992), or in blocking intimal smooth muscle cell proliferation induced by endothelial denudation (Clowes & Karnowsky, 1977).

In this report we compared the effect of heparin in two types of chronic inflammation associated with collagen deposition, the bleomycin and silica-induced pulmonary

fibrosis. Heparin was found to inhibit the bleomycin but not the silica-induced fibrosis, a selective effect which seems to be related to involvement of two different types of fibroblasts in these diseases.

Material and methods

Animals

CBA/J and CBA/J x C57 Bl6 F1 female mice, weighting about 20 g, were purchased from Jackson Laboratories and instilled with bleomycin or silica when about 3 months old. Lewis rats, weighing about 200 g, were obtained from Charles River.

Bleomycin and silica instillation

Bleomycin was purchased from Sigma (St Louis, 1.2–1.7 U/mg) and diluted in saline. Silica (DQ 12, particles <5 μm) were a kind gift from M. Reiser, Steinkohlenberg-bauverein, Essen, FRG, and a suspension of about 20 mg/ml was used. For intra-tracheal instillation in mice, the trachea was exposed under general anaesthesia, elicited by ketamine (150 mg/kg) and xylazine (10 mg/kg), and the desired dose of bleomycin (0.04–0.08 U) or silica (2 mg) was injected in 0.1 ml . Rats were intra-tracheally instilled with 1 U of bleomycin or approximately 6 mg of silica particles in 0.3 ml.

Heparin treatment

Regular heparin and low molecular weight heparin derived from porcine intestinal mucosa were obtained from Sigma (St Louis, H 0880 and H 2149). Mice were treated with a daily injection of 5 units, injected i.m. in 0.1 ml.

Measurement of the lung hydroxyoproline

Determination of hydroxyproline was performed according to described procedures (Huszar *et al.* 1980). In brief, the lungs were submitted to acid hydrolysis and the hydrolysates were neutralized and filtered. An aliquot was then submitted to alkaline hydrolysis and the hydroxyproline concentration determined colorimetrically (Huszar *et al.* 1980).

Antibodies and immunohistochemistry

Mice or rats were sacrificed by opening the aorta under general anaesthesia induced by ketamine (150 mg/kg) and xylazine (10 mg/kg) . The thorax was opened and the lungs were fixed by an intra-tracheal instillation of 10 % formalin. Deparaffinized 4-μm sections were used for immunohistochemistry. Anti desmin, anti vimentin (Bio Tek, Santa Barbara, CA), anti α-actin (Dako, Carpinteria, CA) anti macrophage (ED1 Serotec, Oxford, UK) monoclonal antibodies (mAb) were revealed by a biotinylated horse anti-mouse antibody (BioTek, Santa Barbara,CA) and an avidin–biotin complex tertiary linked to HRP (BioTek) and DAB (BioTek). As a control, mouse IgG was used instead of the mAb. Sections were counter-stained with haematoxylin.

BAL

Mice were sacrificed as before and after opening the thorax, saline was instilled via the trachea under a pressure of about 20 cmH_2O. Fluid (about 2 ml) was recovered by gravity. The liquid was centrifuged to separate the cells from supernatant.

Light or electron microscopy

Mice were sacrificed by opening the aorta under general anaesthesia. After opening the thorax, 2% glutaraldehyde in 0.1 M cacodylate buffer was instilled under 20 cmH_2O pressure. Preparations were examined with a Philips CM 120 electron microscope at 80 kV. Platelets or PMN within the alveolar capillaries were counted as described previously (Piguet & Vesin 1994).

Results

Effect of heparin on the lung hydroxyproline content of bleomycin or silica instilled mice

Instillation of bleomycin (0.04–0.05 U), or silica (2 mg) to mice led, after 15 days, to a significant increase of the lung hydroxyproline content. When mice were treated with heparin, this treatment significantly reduced the lung hydroxyproline content in the bleomycin but not in the silica instilled mice (Table I). A similar effect on the bleomycin-induced collagen deposition was observed using either the regular or the low molecular weight heparin (not shown).

Effect of heparin on the mortality of bleomycin injected mice

Injection of > 0.06 U of bleomycin led to a severe alveolitis with relatively little collagen deposition and an important mortality. Treatment with heparin afforded a significant reduction of the mortality (Table 2).

Table 1. Effect of heparin on the bleomycin or silica-induced collagen deposition

Treatment		Lung h-proline (μg/lung)	BAL	
i-t	i.m.		Cells ($\times 10^3$)	Protein (μg/ml)
None	none	118	5 (2)	101 (24)
Bleomycin (0.04U)	saline	164 (20)	32 (12)	1210 (280)
Bleomycin (0.04U)	heparin	116 (14)*	30 (8)	1100 (320)
Silica	saline	192 (24)	22 (10)	90 (30)
Silica	heparin	195 (33) ns	27 (16)	130 (40)

Results are the mean (s.d.) of the values of a representative experiment involving 6 mice per group, traeted daily with 5 U of heparin and sacrificed on day 15 after instillation.

*Different from the saline treated group by the Mann–Whitney non-parametric *U*-test: $P < 0.02$.

Effect of heparin treatment on BAL

In either the bleomycin or the silica-induced pulmonary inflammation and fibrosis, treatment with heparin had no significant effect on the total number of cells recovered or the protein concentration of the supernatant (Table I). In bleomycin treated mice, the BAL cells were composed of about 74% macrophages and 25 % polymorphonuclear leucocytes, as seen on Giemsa stained cytocentrifuged slides, a partition which was similar in heparin treated mice.

Effect of heparin on histology

Intra-tracheal instillation of bleomycin leads to a patchy alveolitis, made evident by the presence of leucocytes within or close to the alveolar septa, and by areas of alveolar remodelling, disruption of the alveolar architecture and collagen deposition. Study of sections suggests that heparin did not decrease the exudative inflammation but did decrease the area of alveolar remodelling and scarring (not shown).

Instillation of silica led to a peribronchiolar alveolitis, with a tendency to organize itself into nodules. Treatment with heparin had no detectable influence on the extent of these alterations (not shown).

Table 2. Effect of heparin on bleomycin-induced morbidity and mortality on day 15 after instillation

Treatment		Survivors (%)	Body weight (g)
i-t	i.m.		
Bleomycin (0.08 U)	saline	4/16 (25)	–
	heparin	13/19 (68)*	–
Bleomycin (0.04 U)	saline	13/19 (68)	17.5 (0.8)
	heparin	15/19 (78)	19.5 (1.2)†

*Different from the saline injected group with a $P < 0.02$ using Student's *t*-test.

† The mean (s.d.) body weight of the untreated control was 21 (2) g.

Effect of heparin on the cells trapped in the alveolar capillaries as detected by EM

To evaluate a possible effect of heparin on the cells trapped in the alveolar capillaries, random blocks were embedded in Epon and processed for electron microscopy. Cells in the alveolar capillaries were counted. The score, using red blood cells as a standard of blood stasis, indicated that bleomycin increased the number of neutrophils and platelets within the capillaries, as already described (Piguet & Vesin 1994), but that treatment with heparin had no detectable effect on this trapping (Table 3).

Interstitial cells in bleomycin or silica induced pulmonary fibrosis as seen by transmission electron microscopy in mice

In the bleomycin-induced alveolitis, some of the interstitial cells detected in the areas of alveolar remodelling contained lipids and had a cytoplasm rich in filaments (Figure 1A and B). In the silica-induced nodules, the interstitial cells corresponding to fibroblasts were in general devoid of lipid vacuoles or filaments and had a very rich ER (Figure 1C).

Table 3. Effect of heparin on bleomycin-induced platelet trapping

Mice	Platelet	PMN
Non-treated	0.04	0.01
Bleomycin, saline	0.12 (5)	0.04 (2)
Bleomycin, heparin	0.19 (12)	0.07 (3)

Number of cells detected in the alveolar capillaries, expressed as a ratio to red blood cells. Results are the mean (s.d.) of the values observed in 4 mice, sacrificed on day 10 after bleomycin instillation.

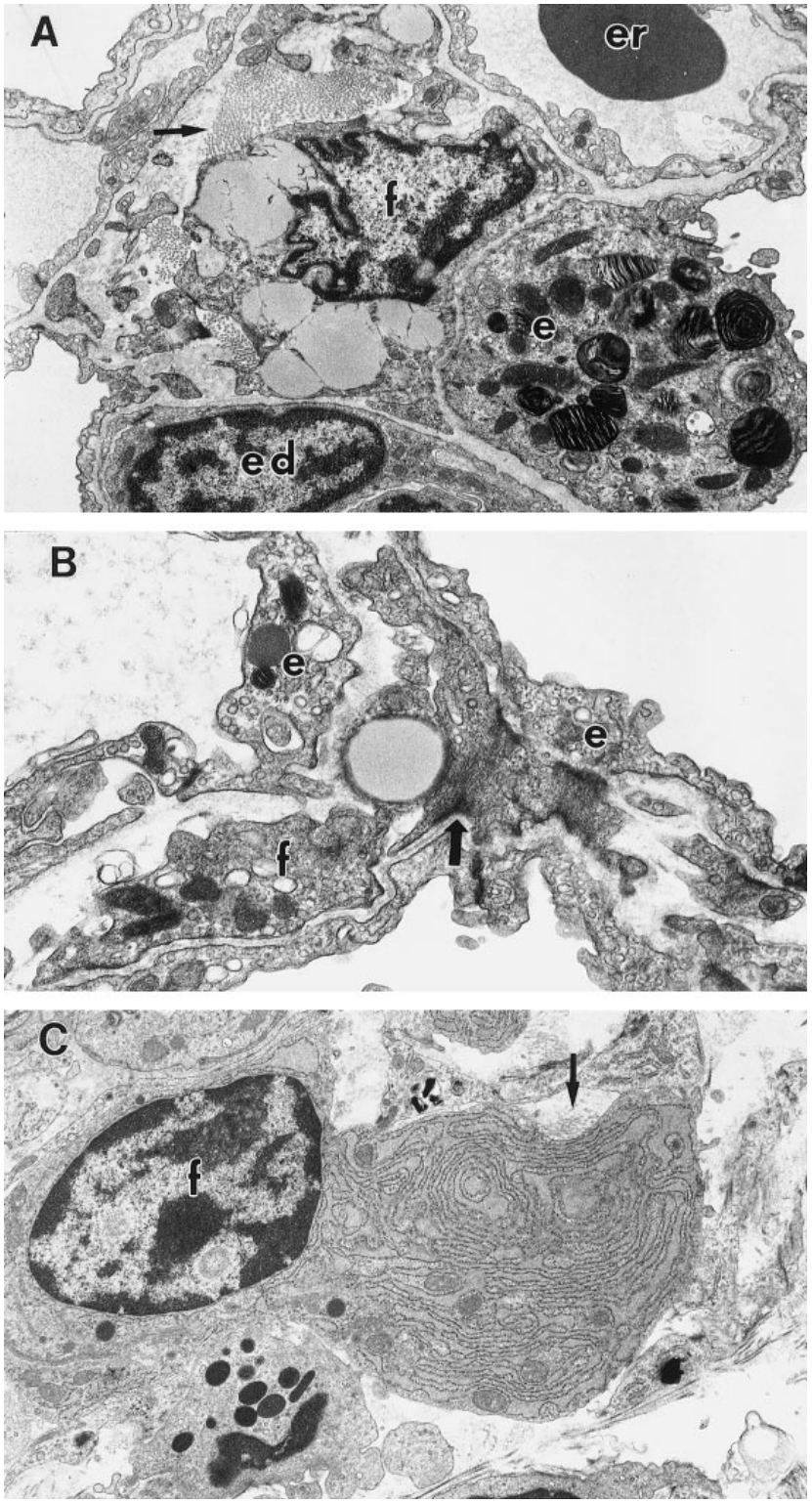

Figure 1. Transmission electron microscopy of the interstitial cells present in the bleomycin (A, B) or silica (C) induced fibrosis in mice. Mouse lungs were isolated 15 days after instillation of bleomycin or silica. In bleomycin, the interstitial cells (f) contain lipid and actin filaments are occasionally visible (B, wide arrow). In a silicotic nodule C, a fibroblast (f) has an extensive RE, and contains neither actin nor lipids. Thin arrow; collagen fibrils, er; erythrocyte, e; epithelial cell, ed; endothelial cell. A, $\times$ 3600; B, $\times$ 8000; C, $\times$ 3800.

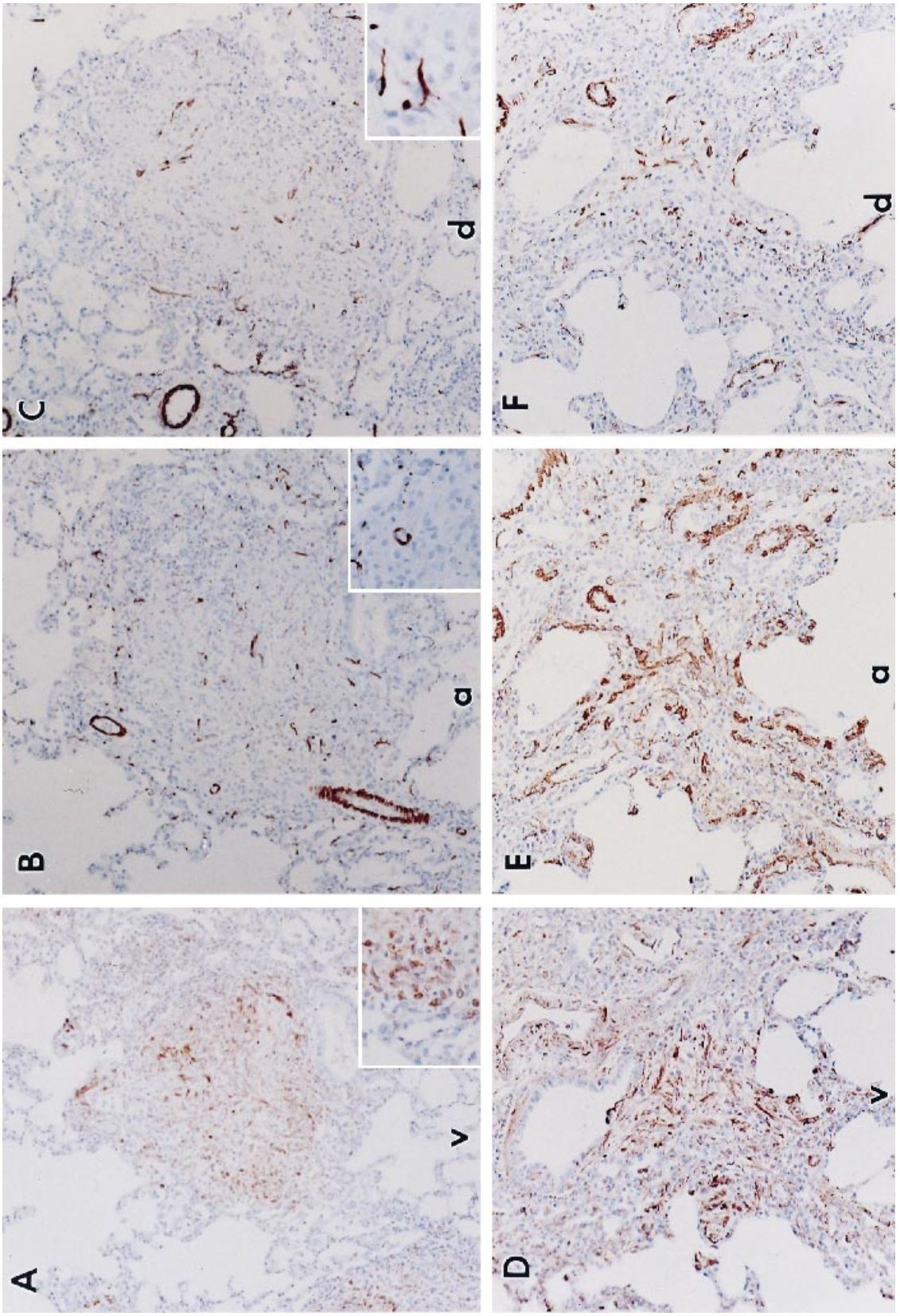

Figure 2. Phenotype of the interstitial cells involved in silica or bleomycin-induced fibrosis in the rat. Sections of lungs from rats instilled with silica (A, B, C) or bleomycin (D, E, F) 15 days before sacrifice have been stained with anti vimentin (A, D), anti α-actin (B, E) and anti desmin (C, F) antibodies. Anti α-actin stained numerous interstitial cells in bleomycin but not in silica-induced fibrosis. In both cases, the majority of interstitial cells stained with anti vimentin mAb, while anti α-actin mAb stained many interstitial cells in bleomycin but not in silica-induced fibrosis. The rare α-actin positive cells in the silicotic nodule had a morphology of pericytes, while the anti desmin mAb stained some rare interstitial cells in both bleomycin and silica-induced inflammation which had the appearance of dendritic cells. (A, B, C $\times$ 40; inserts shows some of the positive cells at a $\times$ 60 magnification).

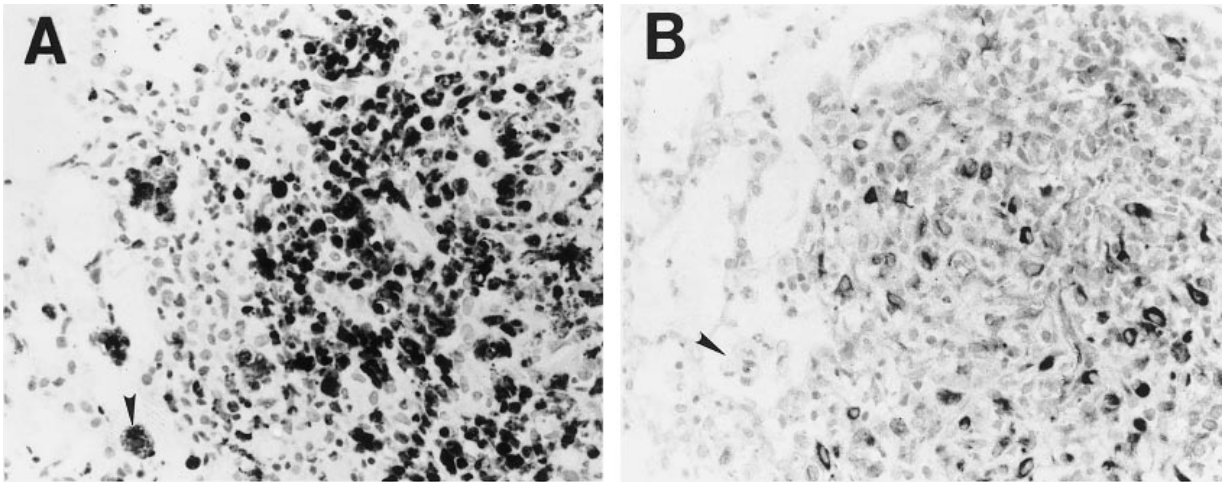

Figure 3. Macrophage and vimentin positive cells in a silicotic nodule. A rat silicotic nodue is stained with anti macrophage (A) or anti vimentin mAb (B). Macrophages are more abundant than vimentin positive cells, both inside the nodule and in the alveolar spaces (arrows); $\times$ 60.

Cytoskeletal markers of the interstitial cells involved in the bleomycin or silica-induced pulmonary fibrosis in rat

It has been reported that the instillation of bleomycin in the rat increases the number of α-actin containing interstitial cells (Mitchell, *et al.* 1989; Vyalov *et al.* 1993). In mice, the anti α-actin mAb stained the smooth muscles of the wall of bronchi or blood vessels, but not the area of alveolar remodelling (not shown). This suggests that the interstitial cells of mice express less of the α-actin isoform than do those of the rat. We therefore examined the expression of cytoskeletal markers in an area of fibrosis elicited by bleomycin or silica in rat.

Anti α-actin antibody stained many interstitial cells in the bleomycin, but not in the silica-induced fibrosis (Figure 2B and E). Anti desmin stained only a minority of the interstitial cells in either case, which seemed to correspond to dendritic cells (Figure 2C and F). In both bleomycin and silica-induced fibrosis, anti vimentin mAb stained interstitial cells (Figure 2A and D) which were in general spindle shaped and correspond most likely to fibroblasts or myofibroblasts. Macrophages were numerous both in the area of scarring and in the alveolar space, but they were, in their large majority, vimentin negative. As was evident for a silicotic nodule (Figure 3), macrophages were more abundant inside and in the adjoining alveolar space than were the vimentin positive cells.

Discussion

Bleomycin or silica-induced pulmonary fibrosis are two types of chronic lung inflammation associated with collagen deposition. These diseases share many pathogenetic mechanisms: they are associated with an increased production of TNF-α in the lung and can both be treated with TNF antagonists (Piguet *et al.* 1989, 1990) or with IL-1ra (Piguet *et al.* 1993a). Both diseases can also be effectively treated with anti CD11a mAb (Piguet *et al.* 1993b). In spite of these similarities, heparin has a selective influence on the bleomycin but not on the silica-induced pulmonary fibrosis.

In vitro or *in vivo*, heparin has been reported to exert a wide range of effects which might potentially play a role in pulmonary fibrosis. Study of the histology and of the BAL suggests that the effect of heparin is more anti-fibrotic than anti-inflammatory, since the alveolar exudate and leucocyte exudation were not greatly affected by heparin treatment. Heparin has been reported to influence platelets (Chen *et al.* 1992) but in the present conditions, heparin did not decrease the platelet trapping in the alveolar capillaries which is associated with bleomycin-induced fibrosis.

Heparin has been shown to inhibit the proliferation of smooth muscle cells *in vitro* and *in vivo* (Tajima *et al.* 1995). Its effect on myofibroblasts is less clear since heparin has been reported to inhibit the growth of myofibroblasts *in vitro* but to promote their growth *in vivo* (Demouliere *et al.* 1992). The most likely explanation for the present finding is that heparin selectively inhibits proliferation and collagen synthesis by myofibroblasts but has no effect on the regular fibroblast. The present work indeed indicates that the interstitial cells involved in bleomycin and silica-induced fibrosis are different and correspond to the myofibroblast or the regular fibroblast respectively.

The normal lung contain at least two types of fibroblast (or non-endothelial intersitial cells); the so-called lipid interstitial cell, and the regular fibroblast (Brody & Kaplan 1983; Sappino *et al.* 1990). The lipid interstititial cell also frequently contains actin as seen by histochemistry and EM, and corresponds therefore to a large extent to the myofibroblast. Myofibroblasts might express the α-actin isoform when fully mature or activated (Adler *et al.* 1989), as is the case during bleomycin-induced inflammation in rat. This might however not be a general differentiation character since in mice, the bleomycin-induced fibrosis does not contain α-actin positive interstitial cells. In this latter condition, the interstitial cells belong nevertheless to the myofibroblast family, as evidenced by their actin content detected by EM (Figure 1). Bleomycin-induced fibrosis is known to be associated with an increase in the number of myofibroblasts and there is evidence that myofibroblasts are the main producers of collagen in this condition (Zhang *et al.* 1994). Pneumoconiosis has been less investigated in this respect and the present study indicates that silicotic nodules contain mainly an 'ordinary' fibroblast, devoid of actin or cytoplasmic lipids, but with a rich ER. Both fibroblasts and myofibroblasts involved in pulmonary fibrosis appear to contain vimentin. In accord with our finding, the silicotic nodule of the rat has recently been reported to be devoid of α-actin positive cells (Mariani *et al.* 1996).

Thus the most likely explanation for the selective effect of heparin in these fibroses is that heparin inhibits proliferation and collagen synthesis by myofibroblasts which are those involved in bleomycin fibrosis, while it has no effect on the fibroblasts implicated in silica-induced pulmonary fibrosis.

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