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Rat Alveolar Myofibroblasts Acquire α-Smooth Muscle Actin Expression during Bleomycin-Induced Pulmonary Fibrosis

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The majority of fibroblasts in alveolar septa are characterized by the presence of cytoplasmic bundles of microfilaments that contain cytoplasmic actin isoforms; these cells have been named contractile interstitial cells or V-type myofibroblasts. In the rat, they express desmin as intermediate filament protein. In this study, we explored the possibility that modulation and replication of such septal fibroblasts result in the appearance of α-smooth muscle (α-SM) actin-positive myofibroblasts, typical of lung fibrosis. Experimental pulmonary fibrosis was produced by a unique intratracheal instillation of bleomycin to 28 rats. Eight additional rats used as controls received the equivalent volume of saline. Paraffin and frozen sections of lungs were examined at days 1, 3, 5, and 7 after treatment. Microfilaments and intermediate filaments were stained using antibodies against total actin, α-SM actin, desmin, vimentin, keratin, and SM myosin. Electron microscopic labeling of desmin and α-SM actin using immunogold technique was done on Lowicryl K4M resin-embedded specimens. α-SM actin appeared in desmin-positive alveolar fibroblasts as early as 24 hours after intratracheal bleomycin instillation; the modulation of α-SM actin in these cells was preceded by a lymphomonocytic infiltration of alveolar septa. Twenty-four hours to 3 days after bleomycin administration, a proliferation of alveolar myofibroblasts occurred. Fibrosis with laying down of collagen fibers took place after the above mentioned cellular modifications. Our results support the view that septal fibroblastic cells can modulate into typical α-SM actin-containing myofibroblasts during experimental bleomycin-induced pulmonary fibrosis. In such a modulation a possible role of cytokines, particularly of transforming growth factor-β, is considered. (Am J Pathol 1993, 143: 1754–1765)

Experimental pulmonary fibrosis has been produced in rats by intratracheal or intraperitoneal administration of bleomycin for nearly 15 years.[1–11] During the establishment of such a fibrosis, a significant polymerization of actin occurs in fibroblastic cells;[4] although the total actin content of the lung changes only slightly, actin-containing contractile cells accumulate[2,3,11] and the contractility of the alveolar tissue increases considerably.[9] More recently it has been shown that the proliferating cells in the alveolar interstitium contain α-smooth muscle (α-SM) actin[5] as do most myofibroblastic cells during wound healing and fibrocontractive diseases.[12] However, whether these α-SM actin-labeled cells correspond to true alveolar myofibroblasts or to pericytes is not established. Furthermore, such α-SM actin-positive cells have been shown to occur in highly remodeled fibrotic rat lungs,[11] whereas the initial events as well as the origin of α-SM actin-positive cells have not been studied.

The aim of this study was to investigate the phenotypic modulations of alveolar interstitial cells during the early phases of bleomycin-induced experimental pulmonary fibrosis. In normal lungs, these cells are equipped with ultrastructurally visible stress fibers, but do not express normally α-SM actin; hence they correspond to V-type (vimentin-cytoplasmic actins) myofibroblasts.[13] Thus, the main criterion to evaluate the alveolar interstitial cell modulation was based on α-SM actin expression, which is known to represent a reliable differentiation marker of myofibroblastic

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cells.[14] To study the very early phases of bleomycin-induced pulmonary lesions before actual fibrosis, electron microscopy using immunogold technique was used. Furthermore, the modifications of other cytoskeletal protein expression in alveolar cells, concerning which there are many controversies in the literature,[2,3,6,8] were investigated. Our results support the assumption that α-SM actin expression is induced in alveolar myofibroblasts before any noticeable fibrotic change.

Materials and Methods

Experimental Design

Twenty-eight female Wistar rats received intratracheally a single dose of 0.75 U/100 g body weight of bleomycin (Lundbeck A.G., Zurich, Switzerland) in 0.2 ml of saline. Eight additional rats used as controls received the equivalent volume of saline only. These instillations were performed through a tracheotomy in anesthetized rats. Seven experimental and two control rats per group were killed by intraperitoneal Nembutal, respectively 1, 3, 5, and 7 days after the intratracheal instillation. The lungs of 12 experimental and four control rats were expanded by intratracheal gelatin[15] before fixation or freezing; those that were not expanded by gelatin were fixed in a vacuum chamber to obtain unfolding of alveolar tissue. Samples were taken for electron microscopy (including immunogold), routine histology, and immunohistochemistry investigations. For histology, the specimens were cut on a rotating Reichart microtome and stained with hematoxylin and eosin (H&E), Goldner's trichrome, and PAS stains. For immunofluorescence, the samples were frozen by liquid nitrogen and stored in a deep freeze at −70° C. Semiserial sections were then cut on a Leitz cryostat and stained with different antibodies (see below).

Immunoperoxidase Staining

Tissue samples were fixed in Methacarn (methanol, chloroform, acetic acid), then embedded in paraffin and processed as described elsewhere.[13] The presence of total actin and α-SM actin was investigated by means of the avidin-biotin complex peroxidase method using a polyclonal anti-actin antibody[16] and a monoclonal antibody against α-SM actin (anti-α-SM-1).[17] Selected sections were also stained by anti-desmin (Dako A/S, Glostrup, Denmark), anti-vimentin (Dako), anti-SM myosin,[18] and anti-keratin (Becton Dickinson, Münchenstein, Switzerland) antibodies. The peroxidase activity was revealed by 3-amino-9-ethylcarbazol (Sigma Chemical Co., St. Louis, MO). Control sections were stained using mouse nonimmune IgG or by omitting the primary antibody.

Immunofluorescence Staining

Cryostat sections (4 to 5 μ thick) were fixed in acetone at −20°C for 5 minutes then air-dried at room temperature for 2 hours and processed as described elsewhere.[13] Double immunofluorescence was performed using anti-α-SM-1 antibodies and a rabbit polyclonal anti-desmin antibody.[14] For the double staining in the second step, we used tetramethyl-rhodamine isothiocyanate-conjugated goat anti-mouse IgG2a (Nordic Immunological Laboratories, Tilburg, The Netherlands) and fluorescein isothiocyanate-conjugated goat anti-rabbit IgG1 (Cappel Laboratories, Cochranville, PA). Thus, α-SM1 actin was revealed by rhodamine and desmin by fluorescein. To minimize cross-reactions, the second antibodies were passed on solid immunoabsorbants of rat serum cross-linked with glutaraldehyde. Control staining was performed using nonimmune IgGs at the same dilution used for the primary antibody. The preparations were examined by a Zeiss Axiophot microscope equipped with epi-illumination and specific filters for rhodamine and fluorescein (Carl Zeiss Inc., Oberkochen, Germany). Photographs were taken using Plan Neofluar 20×/0.5 and 40×/0.90 objectives on T-max 400 Kodak black and white film.

Immunogold Staining for Electron Microscopy

For electron microscopic studies using immunogold technique to reveal α-SM actin and desmin, rat lungs were fixed in 2% paraformaldehyde and 0.5% glutaraldehyde (Merck ABS, Basel, Switzerland) in 0.1 M phosphate buffer. The fixation was performed either by intratracheal instillation of the fixative at 20 cc H_2O or by immersing the specimen in a vacuum chamber at −20 cm H_2O negative pressure. The specimens were chopped into small cubes, left in the fixative for a further 12 hours, and then dehydrated through graded ethanols at decreasing temperatures up to −35°C in a LTE 020 Balzers apparatus (Balzers Union, Balzers, Liechtenstein); they were then maintained at this temperature all through the subsequent infiltrations with Lowicryl K4M resin (Chemische Werke Lowi, Waldkraiburg, Germany).

Polymerization was conducted under UV light first at 35°C for 24 hours then at room temperature for an additional 48 hours. Thin sections were collected on Formvar-coated nickel grids covered by a carbon film. These grids were first incubated for 30 minutes at room temperature in phosphate-buffered saline (PBS; 150 mM NaCl in 10 mM phosphate buffer, pH 7.2) containing 5% normal goat serum (Aurion, Wageningen, The Netherlands), 0.8% bovine serum albumin (BSA) (Fluka Chemie A.G., Buchs, Switzerland), and 0.1% gelatin (Cold Water Fish Skin gelatin 40%) (Aurion), then for 2 hours on drops of anti-α-SM-1 diluted at 1:10 in PBS containing 1% normal goat serum, 0.8% BSA, and 0.1% gelatin and rinsed three times for 10 minutes in PBS containing BSA and gelatin. Other sections were treated in the same way using a polyclonal anti-desmin antibody.[14] The second incubation was performed with goat anti-mouse (or anti-rabbit for desmin) IgG conjugated to 0.8 nm gold particles (Aurion) diluted at 1:100 in PBS containing normal goat serum, BSA, and gelatin for 2 hours at room temperature. They were rinsed three times for 15 minutes in PBS containing BSA and gelatin, three times for 5 minutes in PBS, and finally in sterile distilled water. Amplification of the gold staining was performed by reaction with colloidal silver (Aurion) for 15 minutes at room temperature.[19]

Double immunogold staining for α-SM actin and desmin was conducted by incubating first with anti-α-SM-1 and polyclonal anti-desmin[14] antibodies for 1 hour. For α-SM actin, the second incubation was performed with goat anti-mouse IgG conjugated to 10 nm gold particles (EM-10 nm, Aurion) diluted at 1:10 and, for desmin, with goat anti-rabbit conjugated to 15 nm gold particles (EM-15 nm, Aurion) diluted 1:10 for 1 hour. On these double immunogold-labeled sections, no amplification of gold staining was performed; hence on EM micrographs, particles appear smaller than in separate labeling of desmin and α-SM actin (see Figure 15).

All grids were counterstained with uranyl acetate and lead citrate and examined by a Philips 400 electron microscope.

Results

All the experimental and control rats, except two survived to the intratracheal administration of bleomycin or saline. However, some rats had to be placed in an oxygen chamber for about 15 minutes.

On gross examination, in animals killed at 1 and 3 days, control and bleomycin-treated lungs appeared edematous; later the control lungs became normal, whereas those of rats treated by bleomycin presented on the cut surface pale, ill defined compact areas. Specimens for histology and electron microscopy were taken from such areas.

Histology

The lesions were plurifocal and disseminated in both lungs. They appeared as ill defined, partially condensed areas that were crossed by dilated alveolar ducts (Figure 1). Already 1 day after bleomycin, a manifest lymphomonocytic infiltration of alveolar tissue occurred (Figure 2). In the air spaces few polymorphonuclear leukocytes mixed with occasional desquamated epithelial cells and alveolar macrophages were visible. At 3 days, an occasional mitotic figure could be observed in an interstitial or intra-alveolar cell (Figure 3). Five days, but particularly 7 days after intratracheal bleomycin, alveolar septa became thickened, containing fibroblastic as well as inflammatory cells; they were lined occasionally by type II epithelium. With the trichrome stain, a clear increase in collagen fibers could be observed. In some areas, the cell proliferation from alveolar septa encroached on the alveolar lumen, partially obliterating the air spaces (Figure 4).

Immunohistochemistry

Immunoperoxidase Staining

In control lungs, the alveolar interstitium contained some desmin-positive cells; moreover, besides the vascular and bronchial musculature, only alveolar ring muscle cells and pericytes stained with α-SM actin antibody.[13] Already 24 hours after bleomycin administration, occasional α-SM actin-positive cells appeared in the interstitium. Such cells were distributed focally and could be distinguished from pericytes by their location in the middle of an alveolar septum; they had irregular, tentacular projections insinuating between the capillary lumens (Figure 5). The number of these α-SM-positive alveolar myofibroblasts increased with time: after the 5th day practically all thickened alveolar septa contained such myofibroblasts (Figure 6), which were also stained by anti-desmin antibody. At the 7th day, irregular large bundles of α-SM actin-positive cells were visible; in the alveolar septa, many thin tentacular projections of such cells insinuated between capillaries. Moreover, in the alveolar lumens there were fascicles of spindle cells, several of which were stained with the anti-α-SM-1 antibody (Figures 7 and 8).

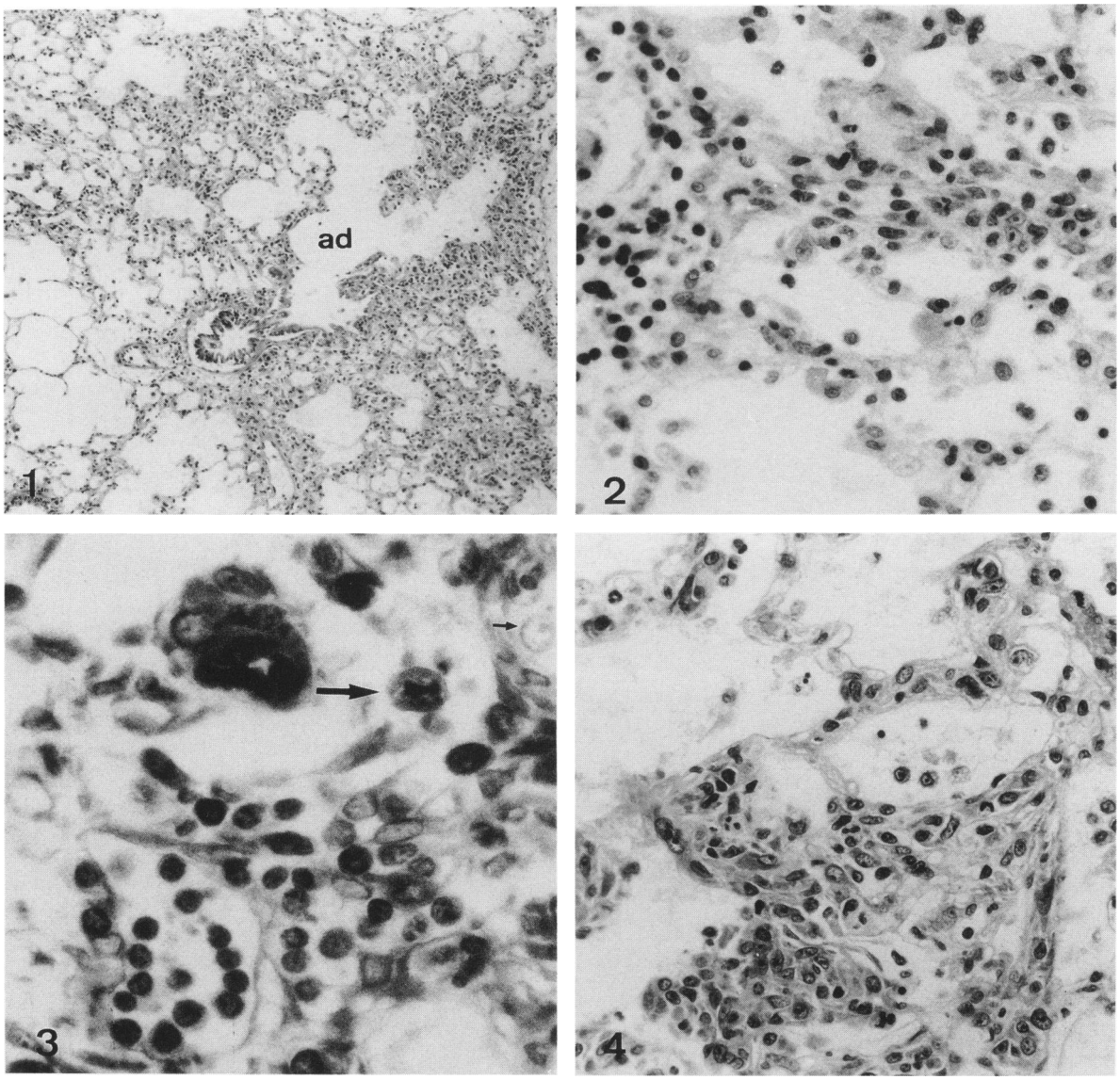


Figure 1. *Bleomycin-treated rat lung; 24 hours. Note the local condensation of alveolar tissue around an alveolar duct (ad). Infiltration of the interstitium and partial collapse of alveolar tissue are already visible on this magnification (H & E, ×40).*
Figure 2. *Same lung as Figure 1 to show dense lymphomonocytic infiltration of alveolar septa (H & E, ×640).*
Figure 3. *Bleomycin-treated rat lung; 3 days. Note a mitotic figure in a mesenchymal cell (thick arrow). There is a monocytic infiltration of the interstitium. The thin arrow points to a nonstained epithelial cell. (vimentin staining ×1300).*
Figure 4. *Bleomycin-treated rat lung; 7 days: Note the thickened and remodeled alveolar septa associated with interstitial cell proliferation encroaching upon the lumen. Together with this fibroblastic cell proliferation, there is a lymphomonocytic infiltration (H & E, ×640).*

Myosin staining was negative for alveolar myofibroblasts; only bronchiolar, vascular, and alveolar ring muscle structures were stained by this antibody in control lungs as well as bleomycin-instilled rat lungs (data not shown). Staining for vimentin was strongly positive in all alveolar interstitial cells as well as those fibroblastic cells proliferating in the alveolar lumen at 7th day after bleomycin administration. As expected, in control and bleomycin-treated lungs, staining for total actin was positive in alveolar interstitial cells and keratin-stained bronchial epithelium as well as some lining cells of alveolar septa (presumably type II epithelial cells).

Immunofluorescence Staining

As shown previously[13] normal alveolar tissue of the rat contained desmin-laden interstitial cells. Such cells appeared much more numerous with immunofluorescence than with immunoperoxidase staining. The same cells were negative to α-SM actin. One day after intratracheal instillation of bleomy-

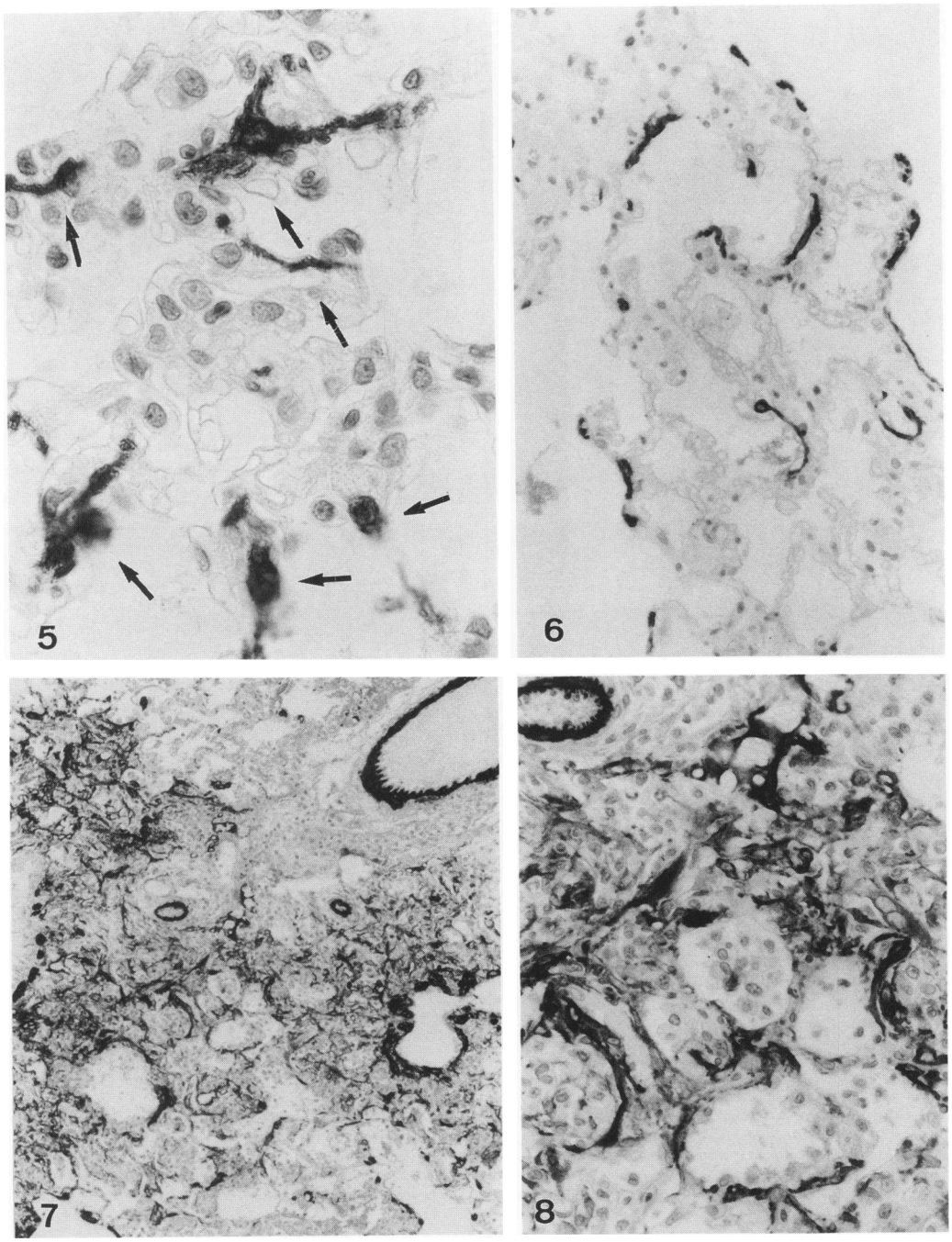


Figure 5 to 8. *Immunoperoxidase staining with α-SM-1 of rat lungs treated with bleomycin.*
Figure 5. *24 hours: Presence of many myofibroblasts in the alveolar septa stained with α-SM-1 (arrows) (×1300).*
Figure 6. *5 days: Note the thickened septa with many α-SM-positive cells (×400)*
Figure 7. *7 days: Highly remodeled fibrotic alveolar tissue rich in α-SM actin-positive cells (×40).*
Figure 8. *Same lung as in Figure 7, higher magnification (×640).*

cin, the number of desmin-positive alveolar myofibroblasts appeared increased and a few α-SM actin-positive cells could be identified in the same areas as those containing desmin-positive cells. On the 3rd day, double immunofluorescence staining clearly showed that α-SM actin was expressed in interstitial desmin-positive cells (Figure 9a and b). On the 5th day, and particularly on day 7, the lungs of treated animals showed a noticeable concentration of desmin and α-SM actin-positive cells; in highly remodeled areas, these cells were organized in bundles. Results obtained by means of immuno-

fluorescence for myosin and vimentin were comparable with those reported with immunoperoxidase staining.

Electron Microscopy with Immunogold Staining

In normal alveolar septa of control lungs, cells identified as interstitial fibroblasts were labeled by antidesmin antibody (Figure 10A). These cells contained bundles of microfilaments as described in our original study on contractile interstitial cells (CIC).[15] They were located particularly at the junction of three alveolar septa; their cytoplasmic projections ran between the capillary and alveolar basement membrane. In such cells, α-SM actin staining revealed no labeling.

After 1 day, no change in respect to control lungs was observed as far as desmin labeling was concerned (Figure 10B). However, at this very early stage, perfectly normal looking alveolar septa contained occasional α-SM actin-laden myofibroblasts (Figure 11) and, in the capillaries and interstitium, there were mononucleated cells identified as monocytes. At day 3, few alveolar septa appeared thickened, edematous, with dissociation of collagen fibers. In such septa, typical myofibroblasts could be recognized. These myofibroblasts were intensely labeled by the α-SM actin antibody.

Five and particularly 7 days after bleomycin administration, a remodeling of alveolar tissue occurred. In thickened septa, there were many myofibroblasts labeled for α-SM actin and desmin (Figures 12 and 13). Furthermore, there was some increase in collagen fibers together with edema and distortion of interstitial tissue. In the interstitium, some monocytes and huge tentacular myofibroblasts laden with α-SM actin were observed (Figure 14). Double labeling with different sized gold particles for α-SM actin and desmin allowed to show that both proteins occurred in the same myofibroblasts (Figure 15).

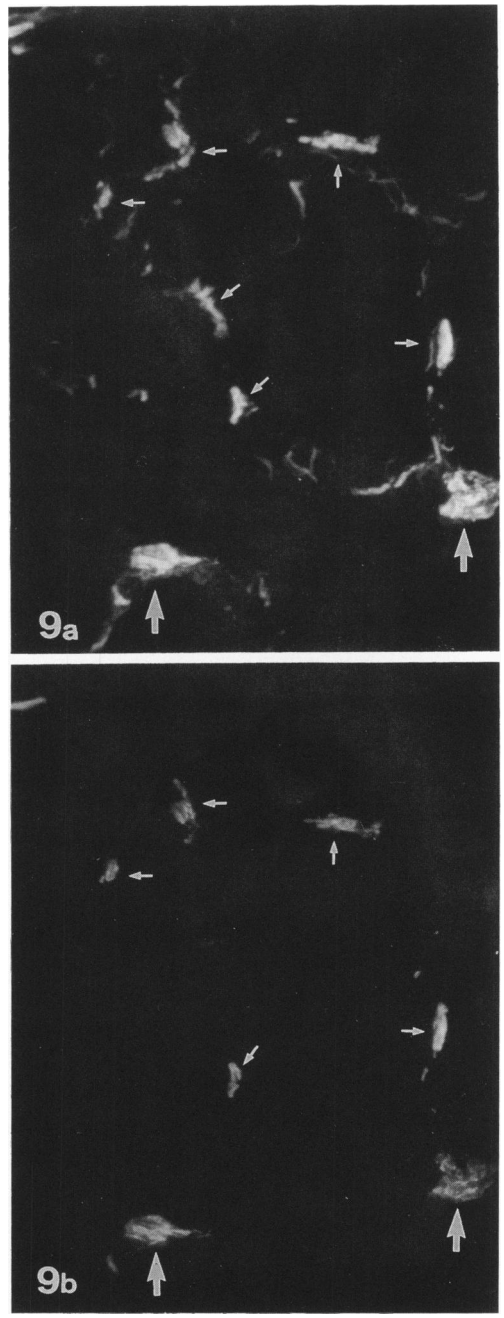


Figure 9. *Immunofluorescence labeling of desmin* (**a**) *and α-SM actin* (**b**) *of bleomycin-treated rat lung at 3 days. This double staining shows that the same cells contain α-SM actin and desmin (thin arrows). The alveolar ring muscles are pointed by thick arrows* (×400).

Discussion

Our study shows that in bleomycin-induced pneumopathy, cellular changes precede fibrosis. Very early in the pathogenesis of pulmonary lesions, that is during the first 24 hours, a focal lymphomonocytic infiltration of alveolar septa takes place; this lymphomonocytosis is associated with the presence of few polymorphonuclear leukocytes and alveolar macrophages in the air spaces. Immediately after this inflammatory reaction, a proliferation of alveolar interstitial cells occurs. Indeed, as early as 24 hours after bleomycin administration, an excess number of desmin-positive alveolar fibroblasts, few of which express also α-SM actin, is visible. In previous studies, a very early occurrence of lymphomonocytic infiltration of alveolar septa has already been pointed out;[1,8,10] on the other hand, it has been claimed that

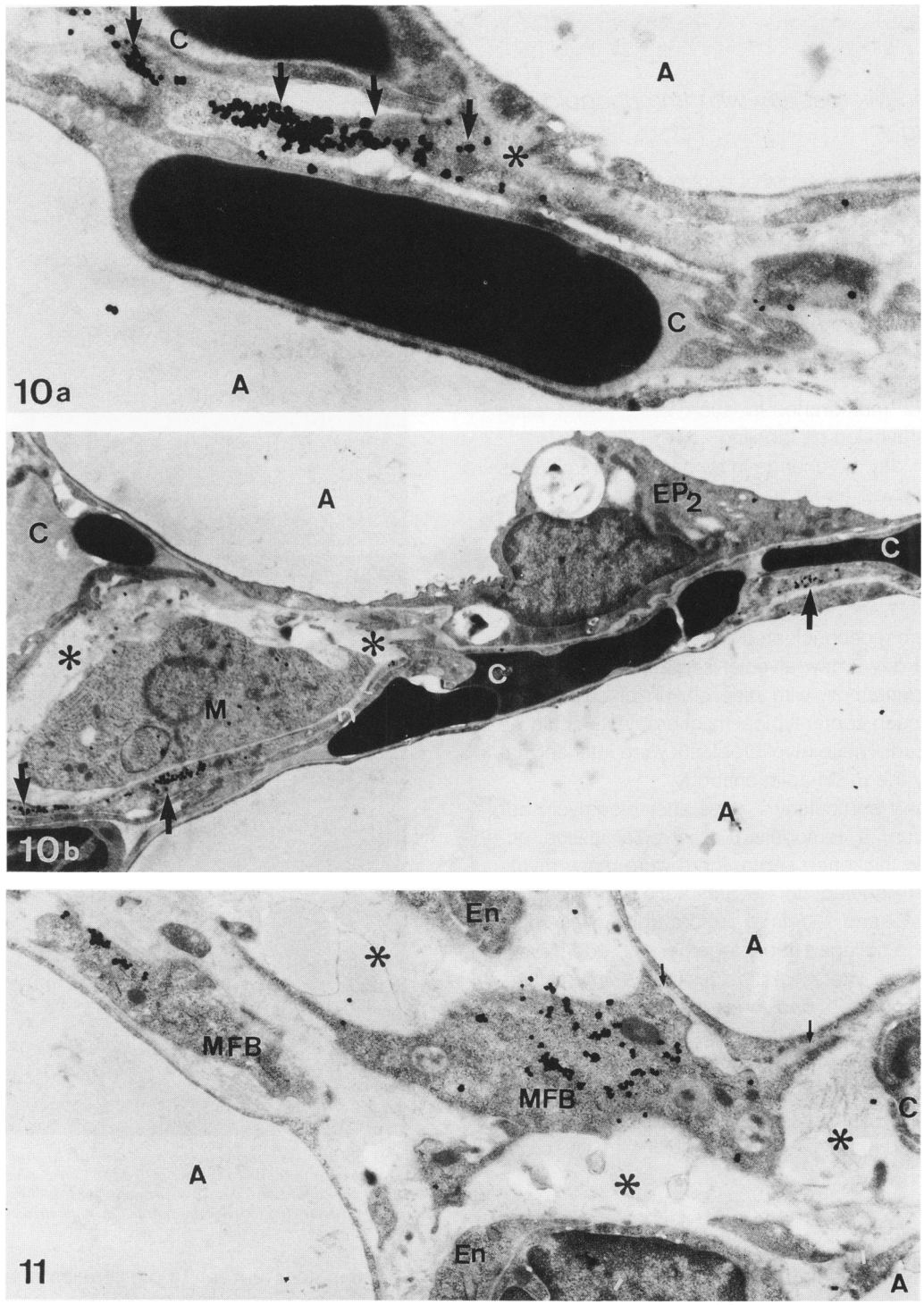


Figure 10. *Desmin labeling with immunogold.* **a**: *Normal rat lung. Note an alveolar myofibroblasts heavily labeled (arrows) running between two capillary (C) lumens. This cell seems to be attached to the alveolar basement membrane (•).* **a**, *alveoli (×15,300).* **b**, *Bleomycin-treated rat lung, 24 hours. Arrows point to desmin-labeled cells. In the interstitium (•), there is a cell, presumably a macrophage (M). C, capillaries; A, alveolus; EP2, epithelium II (×6800).*

Figure 11. *Bleomycin-treated rat lung; 24 hours. An occasional myofibroblast (MFB) is labeled with α-SM1. Note that the cell is located in the interstitium (•), its cytoplasmic projections appearing to be attached to the alveolar basement membrane (arrows).* **A**, *alveoli;* **C**, *capillaries; EN, capillary endothelial cell (×14,800).*

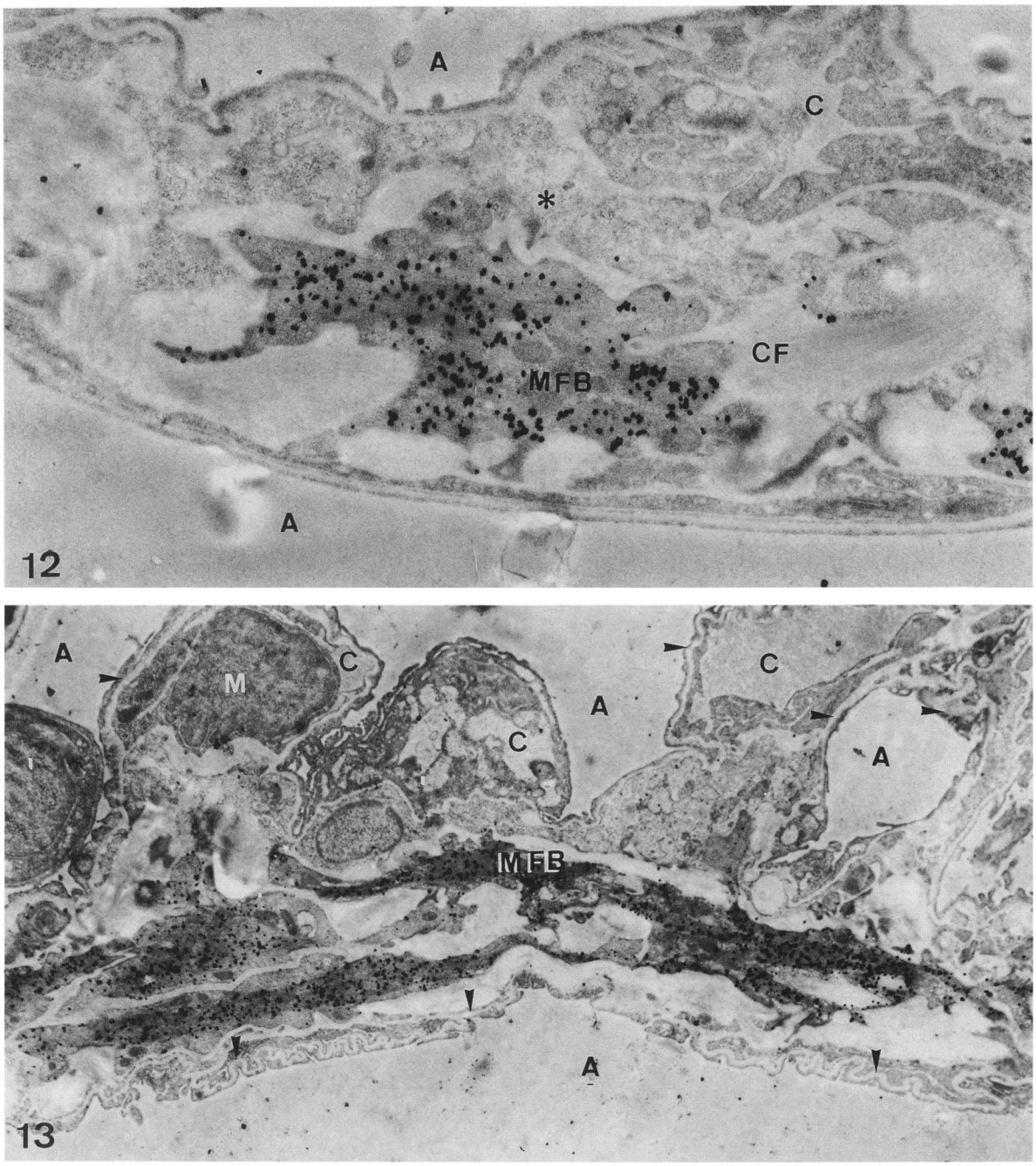
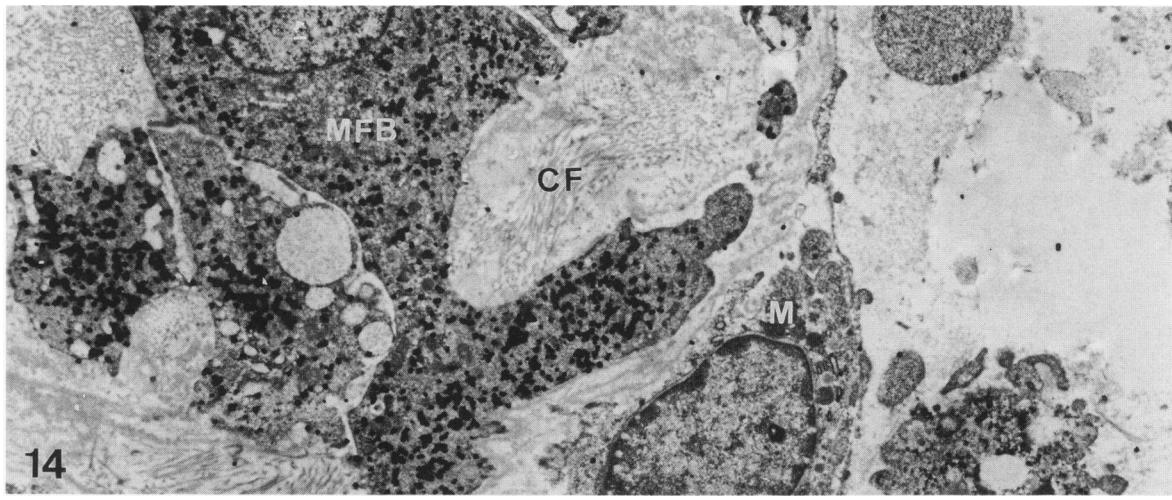


Figure 12. *Bleomycin-treated rat lung; 5 days. Desmin labeling of an alveolar myofibroblast (MFB). This cell is surrounded by collagen fibers (CF). The nature of the cell portion marked by an (*) could not be defined (fibroblast ?).* **A,** *alveolus;* **C,** *capillary (×16,000).*
Figure 13. *Bleomycin-treated rat lung; 7 days. α-SM-1 labeling. Note the presence of a very tentacular myofibroblast (MFB) running into the thick portion of the air-blood barrier. The septum is already fibrotic. Arrow heads indicate alveolar and/or capillary basement membrane.* **A,** *alveolus;* **C,** *capillaries; M, macrophages contained in the capillary (×6400).*

this lymphomonocytic infiltrate is responsible for the local production of fibrogenic cytokines[8] that later on might mediate the collagen production.[20]

Proliferation of actin-containing fibroblasts, ie, CIC (V-type myofibroblasts)[13,15] has been shown in human pulmonary fibrosis[21,22] as well as in bleomycin-induced experimental pneumopathy.[11] Adler et al[11] using morphometric measurements have demonstrated that 14 days after bleomycin administration an increase in the number of CIC, epithelial cells, and particularly of macrophages occurred. Four weeks after bleomycin, the number of actin-laden CIC increased by 10-fold, whereas the number of noncontractile fibroblastic cells seemed



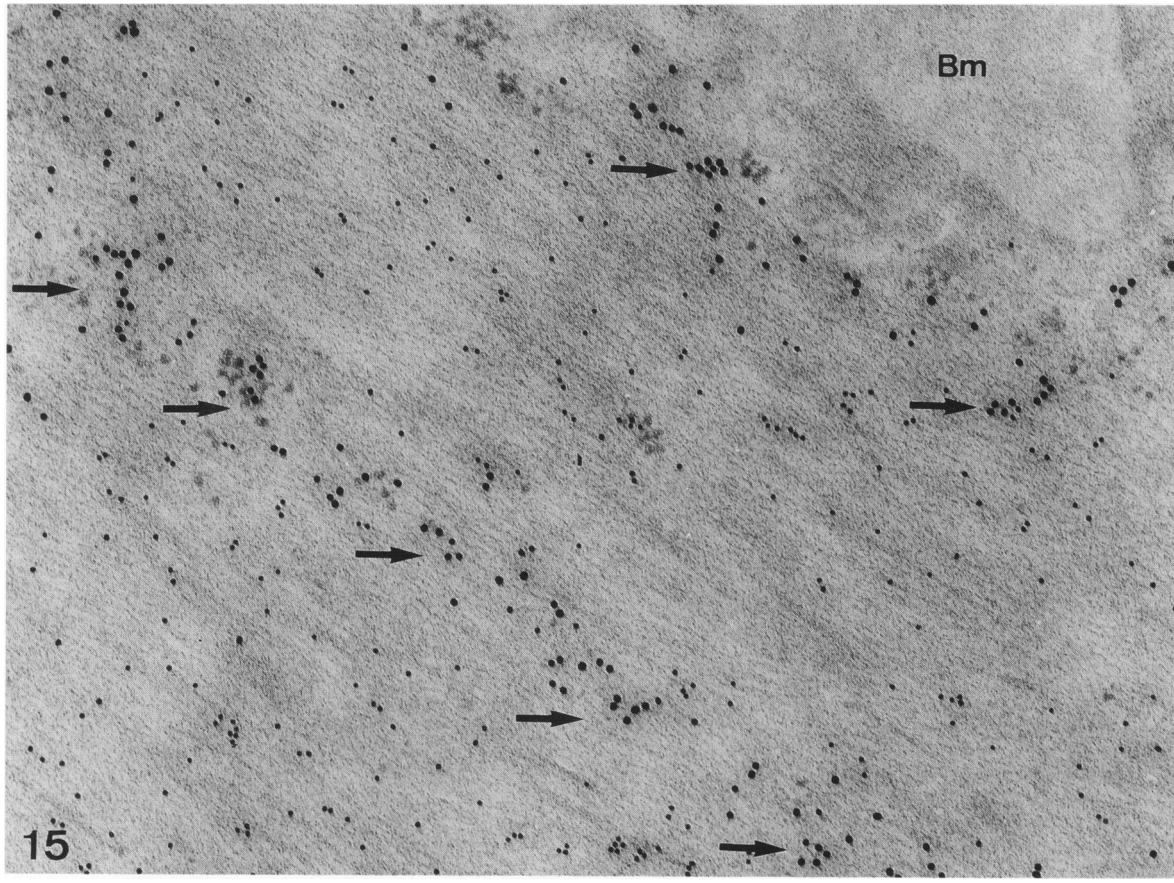


Figure 14. *Bleomycin-treated rat lung; 7 days, α-SM-1 labeling. In a highly remodeled fibrotic area, note the presence of bundles of collagen fibers (CF), myofibroblasts (MFB), and macrophages (M) (×6800).*
Figure 15. *Bleomycin-treated rat lung; 5 days. Double labeling with α-SM-1 and desmin antibodies. Large particles pointed indicated by arrows and arranged in parallel rows correspond with desmin labeling. Tiny particles distributed all over the cytoplasm correspond with α-SM actin. These particles appear smaller than in other figures because they have not been amplified (see* Materials and Methods*) (×65,000).*

to remain unchanged. These investigators concluded that during bleomycin-induced pulmonary fibrosis, the fibroblasts differentiate into CIC.[11] In an earlier study,[4] they had claimed that the increased contractility of alveolar tissue in bleomycin pneumopathy[4] was due to the increased number of contractile cells. Later investigations have shown that between 1 and 4 weeks after intratracheal bleomy-

cin, α-SM actin-positive alveolar myofibroblasts accumulated; such a change occurred in highly remodeled alveolar tissue.[5]

Our current investigation demonstrates that α-SM actin, which is a reliable differentiation marker of myofibroblasts[14] is detected in interstitial cells as early as 24 hours after bleomycin administration. One may, of course, wonder whether such an early occurrence of α-SM actin represents a phenomenon of "modulation" or if it results from a rapid proliferation of a "hidden clone" of interstitial cells. For the following reasons, we favor the modulation hypothesis: We had shown that, in normal human and rat lungs, alveolar CIC contain actin bundles and stress fibers[15] but do not express α-SM actin.[13] In rat lungs these cells are frequently laden with desmin-containing intermediate filaments.[13–15] Hence, during bleomycin-induced pulmonary fibrosis, appearance of α-SM actin in the same cells as those labeled by anti-desmin antibody pleads in favor of the modulation theory. Furthermore, it has been shown in other tissues or organs that α-SM actin is expressed transiently in myofibroblasts during wound healing[14] and that α-SM actin-positive myofibroblasts can exert a mitotic activity.[23] Lastly, a similar modulation of α-SM actin expression has been shown in the perisinusoidal cells of the liver undergoing fibrotic changes;[24] transforming growth factor-β (TGF-β) and tumor necrosis factor-α (TNF-α) have been shown to stimulate the differentiation of such cells into highly activated "synthetic" myofibroblastslike cells.[25]

The only morphological event that apparently precedes the expression of α-SM actin in alveolar myofibroblasts (Figure 5) is the lymphomonocytic infiltration of alveolar septa. It has been shown that such cells produce fibrogenic cytokines such as TGF-β and granulocyte macrophage-colony stimulating factor (GM-CSF), which possess a powerful effect on fibroblast proliferation and collagen synthesis.[20,23,25–28] Indeed Khalil et al[20] have shown that already 2 hours after intratracheal bleomycin administration the TGF-β level of the lungs increases to reach at 7 days a climax of 30-fold compared with that of control lungs. Thereafter, the TGF-β level in bleomycin-poisoned lung extracts decreases up to 28 days, but remains above the control levels. Collagen synthesis, judged by H^3-hydroxyproline incorporation, follows the curve of TGF-β by approximately 1 week delay.[27] According to Khalil et al[20] these findings together with immunostaining of bronchial epithelium by TGF-β antibodies, the increase of TGF-β-laden macrophages, and binding of this cytokine by the extracellular matrix support the hypothesis that TGF-β plays a predominant role in the development of bleomycin-induced pulmonary fibrosis.

At this point, a question arises about the possible role of TGF-β in the modulation of α-SM actin in pulmonary myofibroblasts. Currently, very little is known concerning the relationship between TGF-β and modulation of α-SM actin in myofibroblasts. Mitchell et al[29] have claimed that the addition of TGF-β to growing cultures of lung myofibroblasts, which already express α-SM actin, elevates substantially the expression of this protein. According to their studies, this increase in protein level is not associated with the expression of α-SM actin messenger (m)RNA. Björkerud[30] demonstrated that TGF-β enhances the development of α-SM muscle actin in cultured human arterial smooth muscle cells. In a recent study[31] it has been shown that a subcutaneous administration of TGF-β to the rat results in the formation of a granulation tissue in which α-SM actin expressing myofibroblasts are particularly abundant. Moreover, in such a granulation tissue, the level of α-SM actin mRNA expression is very high. In cultured fibroblasts, such a high α-SM actin level could be induced by TGF-β in the absence of serum, ie, in quiescent cells, and could be reduced by TGF-β neutralizing antibodies.[31]

Do other cytokines play possibly a role in bleomycin-induced pulmonary fibrosis and/or modulation of α-SM actin in alveolar myofibroblasts? Previous studies have shown that TNF-mRNA level increases in the lungs of bleomycin-treated mice 5 days after instillation; this high level is maintained up to 15 days and decreases thereafter.[32] Pulmonary fibrosis—judged by histological examination and also by evaluation of hydroxyproline content—progresses during the first 15 days. Administration of anti-TNF antibodies to bleomycin-poisoned animals prevents the hydroxyproline increase as well as the development of histological lesions. Thus, anti-TNF antibody appears to prevent bleomycin-induced pulmonary fibrosis in mice.[32] However, although TNF-α probably plays a key role in bleomycin-induced pulmonary fibrosis, there is no proof that this occurs as a direct effect of TNF on collagen synthesis or on alveolar interstitial cells.[26] It is indeed conceivable that the production of TNF in bleomycin-treated animals is first followed by diffuse alveolar damage[33] and during the repair other cytokines such as TGF-β, GM-CSF, and platelet derived growth factor (PDGF) would be responsible for the fibrogenic transformations.[6–8,10,26] In any event,

TNF-α does not appear to induce directly the cytoskeletal modulation observed in bleomycin pneumopathy: In experimental granulation tissue, although TNF-α, interleukin-1, PDGF, GM-CSF, and TGF-β produce proliferation of myofibroblastic cells,[34] only TGF-β directly[31] and GM-CSF indirectly[34] are capable to induce α-SM actin expression in these cells.

In earlier studies, the question arosed regarding the origin of contractile cells in pulmonary fibrosis. Among other hypotheses, their pericytic nature has been considered.[13,35] However, this hypothesis is unlikely because in our experimental conditions α-SM actin appears in desmin-containing cells, whereas pericytes are normally desmin negative.[13,35,36] Although we cannot exclude the presence of pericyte proliferation in pulmonary fibrosis, such a phenomenon seems secondary, at least in our model of experimental bleomycin-induced pneumopathy.

The determination of myofibroblastic cell origin is important to understand the mechanism of fibrosis and to devise strategies for its prevention.[37] During skin wound healing, fibroblasts of granulation tissue express transiently α-SM actin.[14] This fibroblastic modulation takes place after the first inflammatory phase and is probably related to the granulation tissue contraction.[12] Our results show for the first time that after bleomycin instillation, an early "neoexpression" of α-SM actin occurs in alveolar interstitial cells. Such cells subsequently replicate and produce an accumulation of extracellular matrix. These results are analogous to what takes place in liver interstitium during the evolution of fibrotic changes.[24] In both cases, interstitial cells stand out by an early α-SM actin expression; they thus acquire a myofibroblastic phenotype and subsequently participate in the induction of fibrosis. In both cases TGF-β appears as a possible candidate to play a role in the induction of α-SM actin expression as it is in wound healing. However, although the basic mechanisms of α-SM actin modulation in granulation tissue development during wound healing and lung fibrosis in bleomycin model may be similar, these two phenomenons appear to differ in their chronology. Other studies are required to determine the exact role of TGF-β in bleomycin-induced pulmonary fibrosis. Such investigations may help to better understand the early phases of the establishment of fibrotic changes in different tissues.

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