The fibrogenic response of adult rat lung to continuous propranolol treatment

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Summary. Fibrogenesis is a common pulmonary response to injury, which is usually preceded by other severe reactions, including inflammation, fluid exudation, and alveolar epithelial damage and proliferation. The purpose of this study was to examine the morphologic effects on the distal lung of a continuous propranolol treatment. Adult male rats were treated, via a subcutaneous osmotic pump, with a continuous (approximately 0.5 mg/hour) dose of propranolol HCl, a potent wide range beta-adrenergic blocking agent, in saline, or saline alone. The animals were killed after one week or three weeks. Electron microscopy of the lungs of the propranolol-treated animals revealed a dramatic increase in the prominence of interstitial cells and fibers of the alveolar septa, along with focal thickening of endothelial cells and some morphologic changes in type II alveolar epithelial cells. In some animals an analysis of total protein content, as well as ^{3}H-proline incorporation into total protein and collagen was undertaken. The results of this study indicated a significant increase in total protein content and proline incorporation into collagen in the lungs of animals treated for seven days with continuous propranolol. There was no evidence of stimulated blood cells, macrophages, edema or severe epithelial damage. This study provides morphologic evidence that continuous treatment with moderate levels of propranolol results in a fibrogenic response in the peripheral lung, in the absence of typical hallmarks of severe pulmonary damage.

Key words: Pulmonary fibrogenesis, Propranolol, Beta-blockade

Introduction

The mechanisms of pulmonary injury and repair have

been subjects of wide interest. A great variety of substances have proven to be injurious to the peripheral lung. These include such air-borne insults as quartz (Sykes et al., 1983), high concentrations of oxygen (Clark and Lambertson, 1971) and paraquat (Smith et al., 1974), as well as blood-borne agents such as bleomycin (Aso et al., 1976), or phorbol myristate (McCall et al., 1983). Despite the myriad of injurious agents, the reactions of the pulmonary tissue to most of them are markedly similar. The typical course, as described by Carrington (1968), includes an initial inflammatory infiltrate, the exudation of fluid into the interstitium and alveolar space, the proliferation of cuboidal alveolar epithelial cells to replace denuded type I cells, and, finally, fibrogenesis, or the deposition of increased connective tissue, particularly type I collagen fibers. This disease course is encountered clinically as the Adult Respiratory Distress Syndrome (Fein et al., 1982), stemming from a variety of direct and indirect injuries to the lung.

Propranolol is a non-specific ß-adrenergic blocking agent which has a variety of actions (see Gerber and Nies, 1985) and is widely used clinically. Propranolol is rapidly cleared by the lung and previous studies in our laboratory have utilized autoradiography to demonstrate substantial specific ß-adrenergic binding in the peripheral lung (Smith and Sidhu, 1984). While a number of workers have demonstrated a ß-adrenergic effect on the pulmonary surfactant system (reviewed Young and Silbajoris, 1985), the effects of beta-blockade on the peripheral lung have not been well addressed. Young and Silbajoris (1985) treated rats with a chronic subcutaneous infusion of propranolol and demonstrated an effect on type II alveolar cell structure, but reported no alterations in the connective tissue compartment of the peripheral lung. Lindenschmidt and Witschi (1985) reported that multiple injections of propranolol elevated total lung collagen in vivo, when administered in concert with a well-defined injurious agent, namely butylated hydroxytoluene, bleomycin or a high concentration of

oxygen. However, they observed no such elevation in collagen when the propranolol was administered alone, to a healthy animal. The present study utilizes an osmotic pump in order to administer a continuous subcutaneous dose, which is 10 times that used by Young and Silbajoris (1985). The results of this study demonstrate a substantial increase in the interstitial connective tissue of the peripheral lung, in the absence of severe damage. We propose this as a useful model of pulmonary fibrogenesis, apparently isolated from substantial epithelial damage, fluid exudation, inflammatory infiltrates, or macrophage aberrations.

Materials and methods

Experimental Protocol

A total of 34 young adult male Sprague-Dawley rats (250-340 g) were weighed and anesthetized with 5 mg/100 g body weight Ketaset I.M. The hair in the interscapular region was shaved and a small (1.5 - 2.0 cm) skin incision was made. An osmotic pump (Alza Inc., model 2ML1 or 2ML4) was implanted in a subcutaneous compartment and the incision closed with sterile surgical staples. In half of the animals the pump contained a solution of DL-Propranolol HCl (Sigma) in normal sterile saline, at a concentration such that the pump released 0.5 mg/hour propranolol. This concentration was dependent on the mean pumping rate of the model, as well as the specific lot of osmotic pumps. Control animals were similarly treated except that the osmotic pumps in these contained only normal sterile saline.

The animals treated with the model 2ML1 pumps were housed individually and fed ad libitum for 7 days in the Wellesley animal quarters. Those treated with 2ML4 pumps were similarly maintained for 21 days. In no case did the animals demonstrate any outward signs of discomfort or respiratory difficulty.

Electron Microscopic Studies

At the end of the experimental period the animals were re-weighed and anesthetized with pentobarbital sodium I.P. The thorax was opened and the upper lobe of the right lung, as well as a significant portion of the lower lobe, was removed and immersed immediately in cold 2% phosphate buffered glutaraldehyde. They were minced and placed in fresh fixative for 2 hours. The tissue was then dehydrated through a graded ethanol series, infiltrated and embedded in Polybed 812 (Polysciences, Inc.). Following polymerization semi-thin (0.5 μm) and thin (60-80 nm) sections were cut using glass or diamond knives on a Sorval MT-1 or MT-5000 microtome. The semi-thin sections were mounted on glass slides and stained with toluidine blue. The thin sections were mounted on 200 mesh copper grids, stained with uranyl acetate and lead citrate, and examined in either a Siemens 1a, or a Phillips CM10 electron microscope.

Analysis of Total Protein and Proline Incorporation

Eight animals were implanted with the one week pumps, as above. On the sixth post-implantation day, they were injected subcutaneously with 200 μCi of ^{3}H-proline (specific activity approximately 140 ci/mmol). On the seventh day they were killed and tissue was obtained, as above. One gram of lung tissue was homogenized in 50 ml of a NaCl - tris HCl buffer. Aliquots were taken from each homogenate and analyzed for total protein content, as well as ^{3}H-proline incorporation. Total protein was determined using a standard (Bio Rad) colorimetric assay. Total ^{3}H-proline labelling was determined by liquid scintillation counting (LSC). ^{3}H-proline incorporation into total, as well as collagenous protein, was performed using a modification of the procedure of Miller and Undenfriend (1970).

Results

At the end of the treatment period the animals treated with propranolol appeared outwardly indistinguishable from the controls. The weights of the two groups were very similar, each showing approximately a 10% weight gain over one week, and a 35% gain over three weeks. Grossly, the lungs of all animals appeared normal and no differences were noted between treated and control animals.

One Week Treatment

Light microscopic observations of 0.5 μm toluidine blue stained sections revealed that the peripheral lung of the saline-treated animals appeared normal in every respect. The lungs of propranolol-treated animals revealed, in some cases, septa which appeared diffusely thickened, but showed no evidence of severe damage, such as epithelial denudation, edema or inflammatory infiltrates.

Electron microscopic observation of the lungs of saline-treated animals revealed no unusual features. The alveolar septa were thin and consisted primarily of capillaries covered by thin blood-air barriers. These were supported by small to moderate amounts of connective tissue, which included collagenous and elastic fibers as well as fibroblasts. These were typically observed as small portions of cytoplasm amidst the fibrillar material, with occasional nuclear profiles.

The principal findings in the lungs of animals treated with propranolol for one week are demonstrated in Figs. 1 and 2. There was an increase in the connective tissue of the alveolar septa. There appeared to be an increase in the number of interstitial cells, which had the characteristics of active fibroblasts. Cytoplasmic extensions of these cells were abundant throughout the interstitial compartment, and usually contained prominent Golgi bodies, vesicles, and dilated rough endoplasmic reticulum. Many of the interstitial cells contained prominent lipid droplets. The matrix of the connective tissue consisted of bundles of type I collagen

Table 1. Total protein and ^{3}H-proline incorporation in the 7-day group.

Total Protein (mg protein / g lung)

Propranolol-treated	56.25 ± 2.19	
Saline-treated	44.37 ± 2.04	(p<.005)

Total ^{3}H-proline Labelling (DPM / 0.02 g lung)

Propranolol-treated	13,697 ± 1548	
Saline-treated	8,259 ± 435	(p<.001)

^{3}H-proline Incorporation Into Proteins (DPM / μg protein)

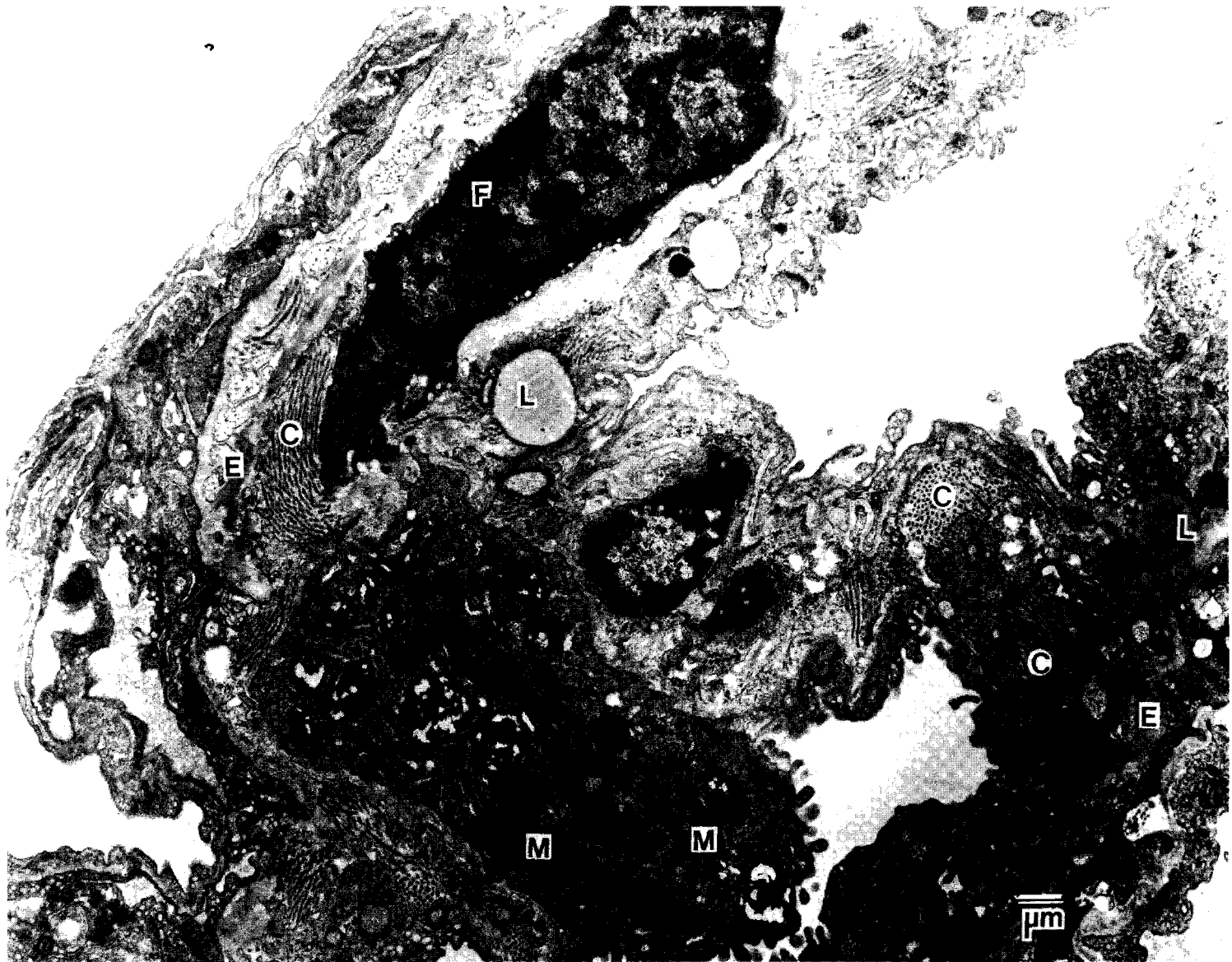|		
Total Proteins		
Propranolol-treated	7629.7 ± 751.0	
Saline-treated	7233.4 ± 516.8	(n.s.)
Collagen		
Propranolol-treated	2425.0 ± 74.0	
Saline-treated	2123.8 ± 112.0	(p<.1)

Fig. 1. Electron Micrograph (EM) of lung tissue from a rat treated with continuous propranolol for 1 week (1 week group). Note relatively thick alveolar septum with fibroblast (F) containing lipid droplet (L). There are fairly abundant deposits of collagenous (C) and elastic (E) fibers extracellularly. The type II alveolar epithelial cells at bottom contains abnormally large mitochondria (M). × 7,300

fibrils and elastic fibers.

In most areas there was some evidence of structural alterations in the capillary endothelial cells. This was often an increase in thickness, and the presence of many vesicles within their cytoplasm. In addition, there was sometimes connective tissue matrix interposed between the endothelial cells and the type I alveolar epithelium, or between the endothelial cell and its basal lamina.

The alveolar type I epithelial cells did not appear altered in any substantial or reproducible manner. Type II cells possessed the normal complement of lamellar bodies and other organelles, but the mitochondria often appeared abnormal. These were sometimes cup-shaped, or elongated and club-like. There was no evidence of edema, platelet margination in the capillaries,

mononuclear infiltration in the interstitium, or an excessive number of macrophages.

The results of the analyses of total protein and proline incorporation into total protein and collagenous protein in the one-week group are listed in table 1. These results demonstrated a significant increase in the total protein content in the lungs of propranolol-treated animals. In addition, there was significantly more ^{3}H-proline labelling in homogenates of these lungs, when compared to those of saline-treated animals. Further analyses demonstrated that while there was no significant difference in ^{3}H-proline incorporation into total protein between the two groups, there was a difference (significant to the 0.1 level) in the incorporation into collagenous proteins.
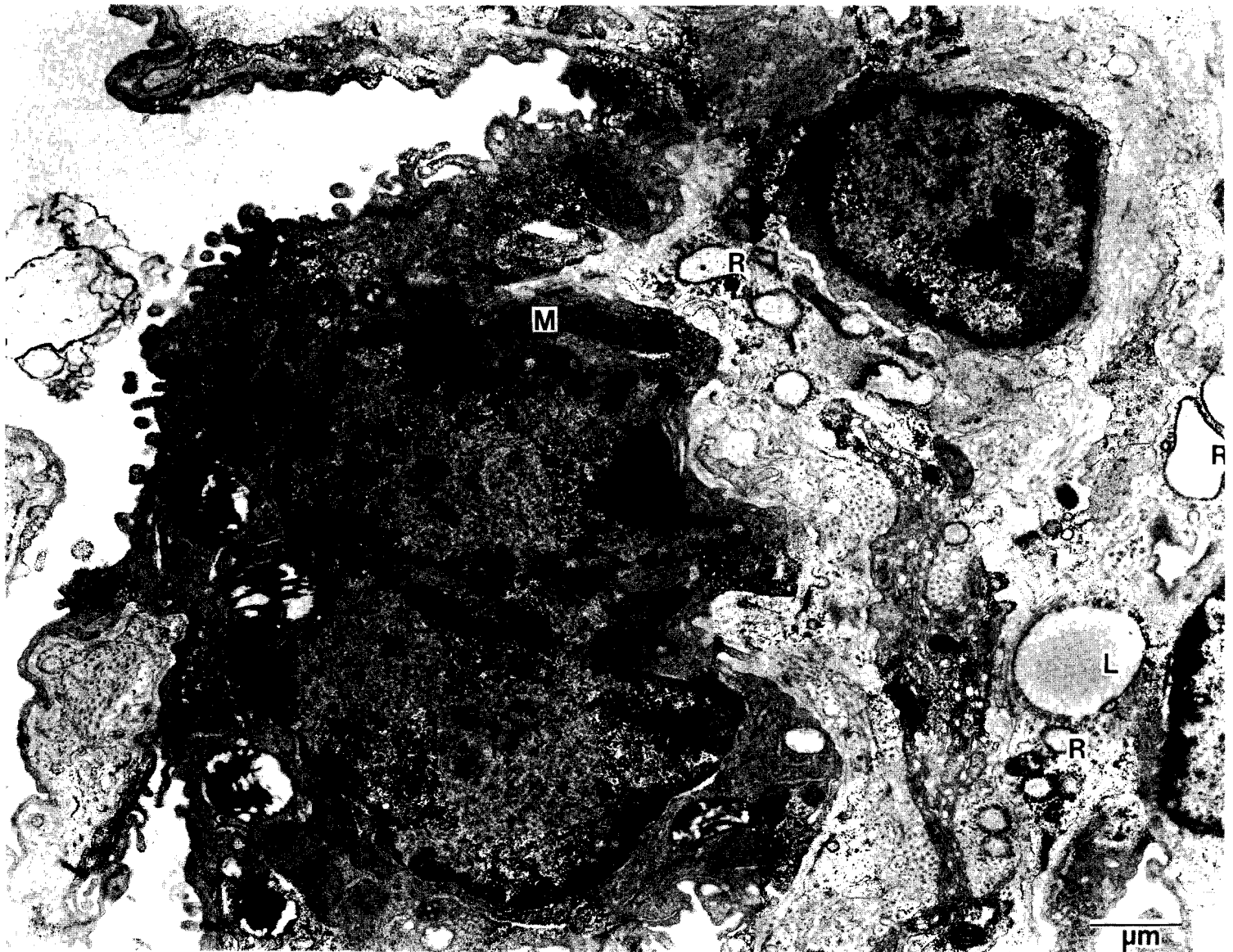

Fig. 2. .EM of lung tissue from a 1 week group rat. Note abnormal mitochondrion (M) in type II cell. Fibroblast cytoplasm in interstitium appears active, with many profiles of dilated rough endoplasmic reticulum (R). A large lipid droplet (L) is also seen. × 14,500

Three Week Treatment

Light and electron microscopic observation of saline-treated animals revealed peripheral lung which appeared normal in every respect. Light microscopic observation of the lungs of animals treated for three weeks with propranolol revealed some areas of thickened septa, with apparently increased cellularity. There were, however, no signs of severe damage.

Electron microscopy revealed obvious differences from control lungs, as demonstrated in figures 3-6. The alveolar septa typically contained large amounts of connective tissue fibrils and cells. There was continued evidence of focal endothelial thickening, as well as some areas of focal thickening of the alveolar epithelium. A common finding was basal laminae which were either absent or very much increased in thickness. This occurred in large areas, and was characterized by collagen fibrils within the basal lamina, or by large areas of connective tissue replacing the basal lamina.

A consistent finding in these lungs were morphologically abnormal mitochondria within the type II alveolar epithelial cells. These appeared at times large and angular or, as was the case in the one week animals, hook-shaped or long and irregular. As in the one-week animals, there was no evidence of an inflammatory infiltrate, edema, or an increase in macrophages. There was also no evidence of alveolar epithelial replacement, although the interstitium appeared more cellular.
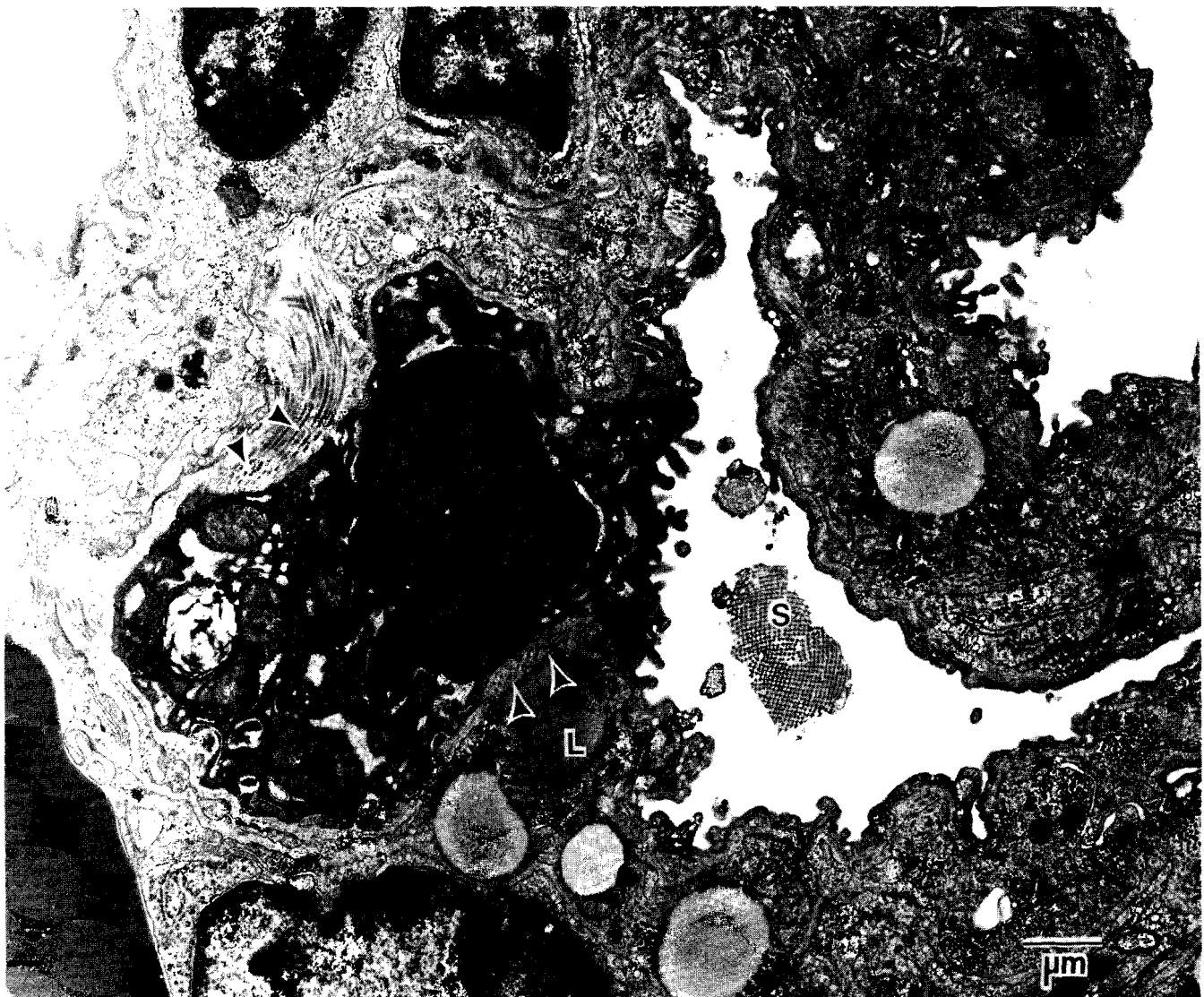

Fig. 3. EM of lung tissue from rat treated with continuous propranolol for three weeks (3 week group supplanting). There are many lipid droplets (L) within the fibroblast cytoplasm. Note collagenous fibers (C) directly under type II cell at arrow, apparently supplanting normal basal lamina. Note relatively thick, cellular alveolar septa. S; pulmonary surfactant in alveolar space. × 12,000
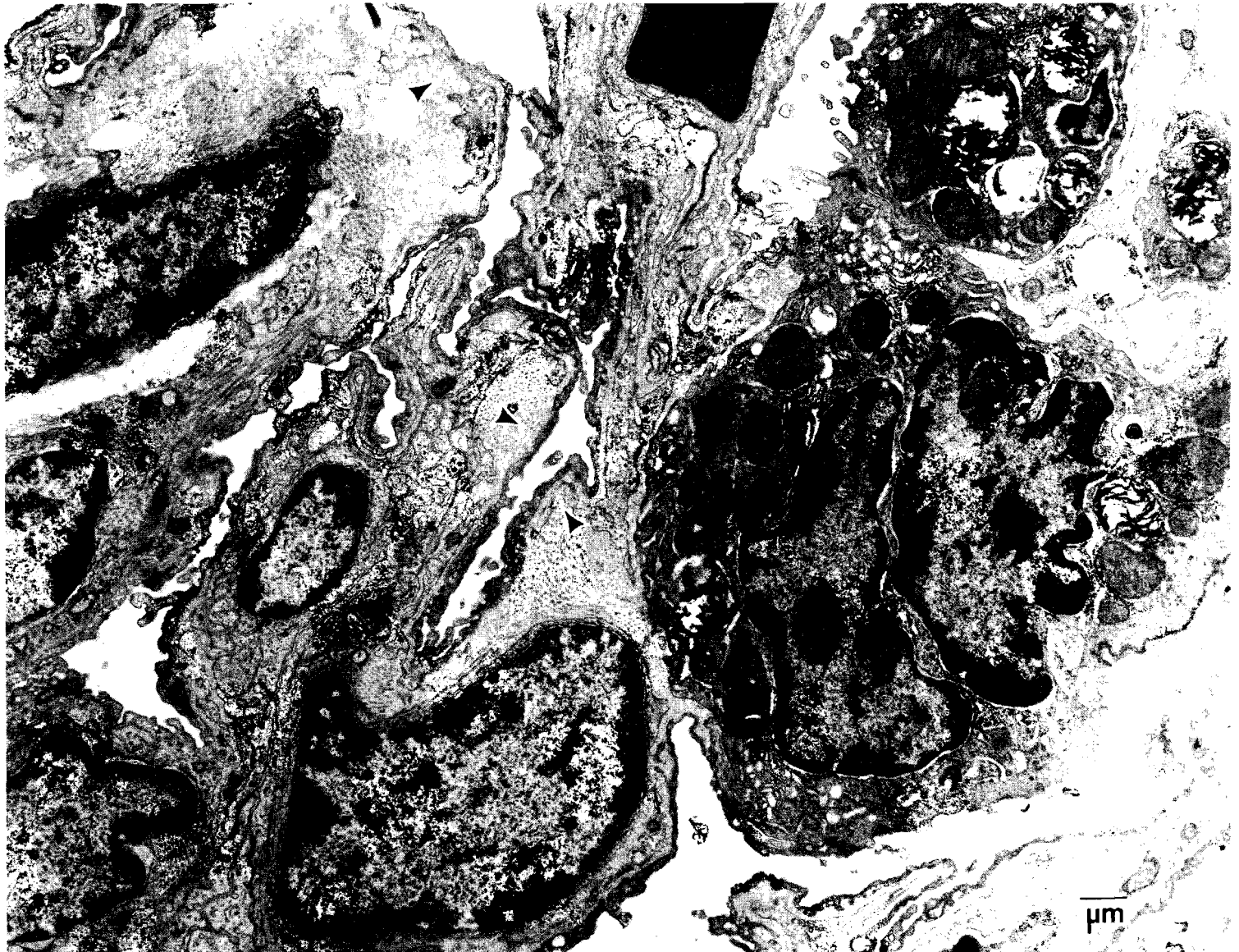
Fig. 4. EM of lung tissue from a 3 week group rat. Note large amounts of collagenous fibrils and areas of fibroblast cytoplasm within relatively thick alveolar septa. The collagen fibrils appear to supplant the basal lamina at some areas (arrows). × 7,800

Discussion

The results of the present study provide strong morphological evidence that chronic, continuous treatment of rats with moderate levels of propranolol results in a fibrogenic response in the peripheral lung. This response is characterized by an increase in interstitial connective tissue cells and fibers. There appears to be some effect on the capillary endothelial cells and on type II alveolar epithelial cells. There was little evidence, however, of the classic hallmarks of pulmonary injury which typically precede connective tissue derangements.

The results of the present study differ from those of Young and Silbajoris (1985), who reported no connective tissue response in the lungs of rats, which were treated

similarly, but a 10-fold lower dose. It should be noted that they also reported no qualitative differences in type II cell morphology after propranolol treatment.

The results of the present study also differ markedly from the reported morphologic findings following typical pulmonary injuries. In contrast to the usual scenario (Carrington, 1968), the lungs of propranolol-treated rats showed no evidence of a granulocytic or lymphoid infiltrate, edema, macrophage concentration, or significant epithelial damage. It is possible that studies utilizing continuous propranolol treatment at shorter time points may reveal early responses which reflect those observed in typical lung injuries. However, initial studies in our laboratory using single bolus subcutaneous injections, or repeated subcutaneous injections over three days, have not revealed signs of inflammation or
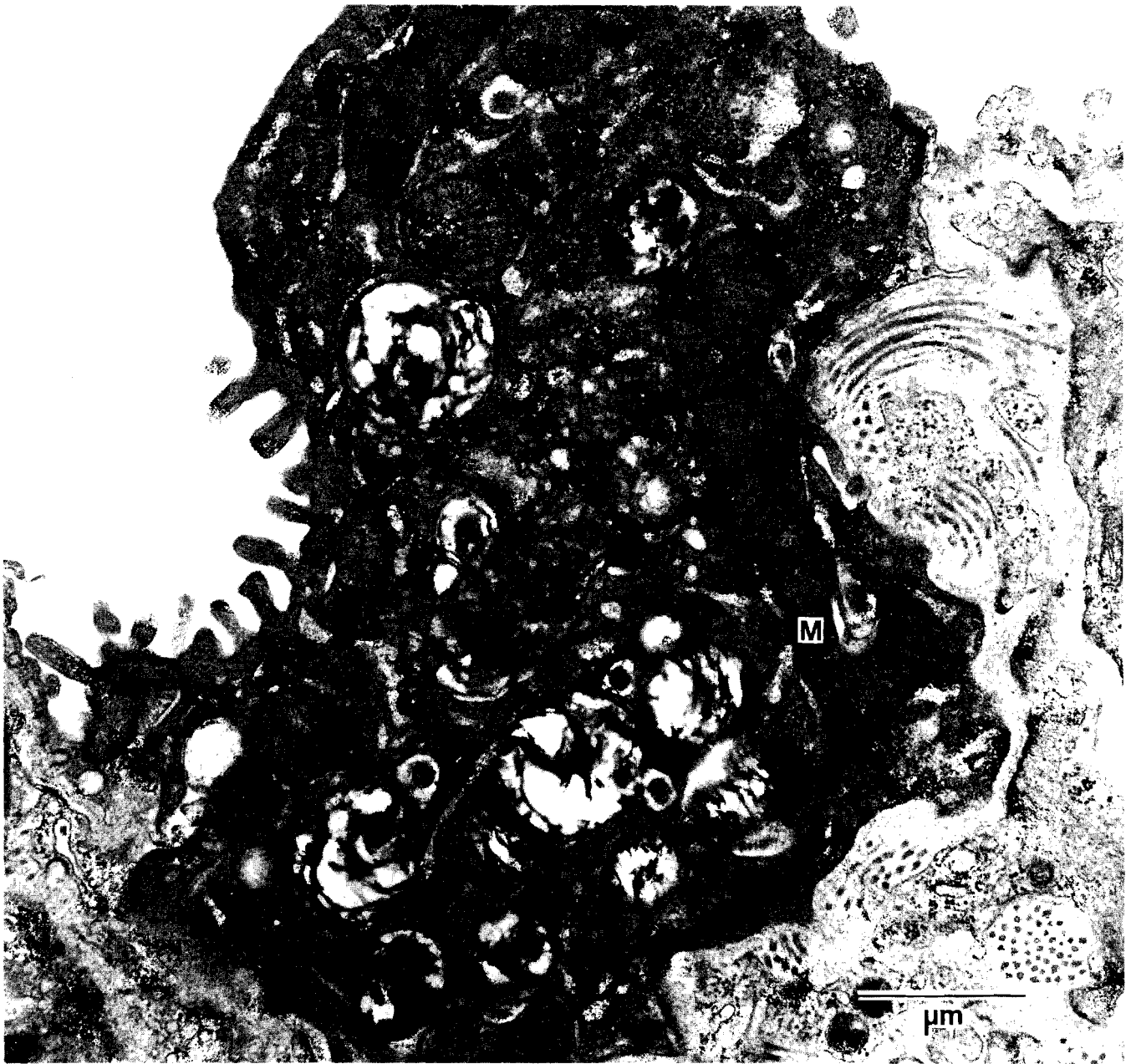
Fig. 5. Type II alveolar epithelial cell from the 3 week group. Note abnormally large, hooked mitochondrion (M). Again, there are collagenous fribrils in the position of the basal lamina. × 28,000

epithelial damage (unpublished observations). In addition, an early inflammatory response, such as that seen in bleomycin-treated animals, would probably be followed by epithelial damage and subsequent alveolar type II cell proliferation, which we did not observe.

Lindenschmidt and Witschi (1985) treated rats with multiple injections of propranolol, in conjunction with a pnuemotoxin and observed increased total collagen in the lungs of animals receiving propranolol. However, it is important to note that they observed no increase in lung collagen in animals treated with propranolol alone, in the absence of a classic lung injury. This points out the

apparent importance of the continuous nature of the treatment in the present study.

The response of the lungs to propranolol shares several features with the morphologic findings of Crapo and co-workers (1978, 1980) in the oxygen-adapted lung. Following seven days of treatment with 85% oxygen, they observed increased interstitial cells and fibers, with little epithelial damage. However, they did observe frank endothelial necrosis and edema, which were not observed in the present study. Another common finding of some interest is the presence of morphologically abnormal mitochondria within type II alveolar epithelial

Fig. 6. A wide blood-air barrier from the 3 week group. The alveolar space (A) is at the top, while the capillary lumen (L) is at the bottom. The usually thin basal lamina has been supplanted by a thick barrier consisting of collagenous fibrils (C), and portions of fibroblast cytoplasm (F). × 18,200

cells in both oxygen-adapted and propranolol-treated rats.

The mechanism of action of continuous propranolol treatment in causing the observed fibrogenic response remains unclear. Previously published brief case reports have indicated that patients have developed pulmonary fibrosis in response to Pindolol (Musk and Pollard, 1979) and Practolol (Erwteman et al., 1977) treatment. These drugs, however, differ significantly from propranolol in that each contains substantial intrinsic sympathomimetic activity, which propranolol does not. Propranolol may be exerting its effect through the control of cyclic nucleotides, as suggested by Lindenschmidt and Witschi (1985). The occurrence of morphologically altered type

II alveolar epithelial cells, as well as apparently damaged endothelial cells, may also be significant. The possible role of these cells becomes important in this model because of the absence of other, more widely accepted cellular mediators of fibrogenesis, namely macrophages, neutrophils, or lymphocytes (as reviewed in Reiser and Last, 1986).

In summary, although many questions remain unanswered, morphologic findings give strong evidence that continuous propranolol treatment, via a subcutaneous osmotic pump, results in a fibrogenic response in the peripheral lung. Although this result may be expected of lungs undergoing a response to acute injury, it is remarkable in this case that increased

collagen deposition occurs in the absence of an observed increase in neutrophils or other cells typically associated with a fibrogenic response. We feel that this model may be useful in the study of pulmonary fibrogenesis, as an event separated from epithelial denudation, or the products of stimulated macrophages or blood cells.

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