Capillary Remodeling in Bleomycin-Induced Pulmonary Fibrosis

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Lung fibrosis is a process in which collagen is laid down and the delicate capillary–alveolar relationship is disturbed. The architectural changes which occur in the capillaries, a main element of the oxygen transferring unit, are difficult to illustrate without a three-dimensional tool, such as scanning electron microscopy. Therefore, a scanning electron microscopic study was undertaken to show the capillary changes of lung fibrosis. Fibrosis was induced in rats by intratracheal instillation of bleomycin. After 30 days the rats were sacrificed, and the vascular tree of the lung was cast with methacrylate. The fibrosis was patchy. The intercapillary space became wider; and some capillaries had large, irregular dilatations. Occasionally giant capillaries (up to 19 μ in diameter) were noted. The pleural and alveolar capillary diameters increased ($P < 0.01$), and the branching frequency decreased ($P = 0.02$). The center of the capillary rings, which has been suggested to be the site of contractile interstitial cells, increased in size ($P = 0.03$). The appearance of irregularly shaped capillaries and an increase in diameter without a change in density of alveolar capillaries, resulting in a loss of surface area and a decrease in branching, are the main scanning electron microscopic findings of the remodeling which occurs in pulmonary capillaries in lung fibrosis. These changes may partially explain the functional derangement of this disease. (Am J Pathol 1986, 125:97–106)

MOST descriptions of the vascular changes occurring in pulmonary fibrosis are of vessels larger than capillaries,[1] because little change has been noted in these structures by light microscopy. Using 10% barium sulfate gel, which does not pass through channels less than 30 sq μ, Turner-Warwick demonstrated the presence of many bronchial vessels communicating with branches of the pulmonary arteries in areas of abnormal lung, in patients dying with pulmonary fibrosis. These vessels were thin-walled, about 50 μ in diameter, and found most often beneath the pleura and at the level of the fourth generation bronchi.[3] Heath and colleagues showed similar plexuses in histologic sections of honeycomb lung.[4] Gracey and colleagues found a decrease in the number of capillaries within alveolar septa and asymmetric interposition of collagen and cells between the surface of the capillaries and alveolar lining cells in areas of fibrosis at autopsy in human lungs.[5] Bignon and colleagues found that the volume of vessels less than 1 mm in diameter was increased and the number of branches was reduced, with diffuse narrowing of distal arteries in areas of fibrosis.[6] From studying the biopsy specimens of 37 patients who underwent open lung biopsy for fibrosing alveolitis, Coalson found a decrease in the luminal size of vessels within remodeled alveolar walls. The endothelial cells were plump, lacking their normal attenuation, and filled with organelles. Pericytes were prominent around the capillaries, and the endothelial basement membrane of affected capillaries was often laminated.[7]

We postulated that major architectural changes occurred in capillaries in lung fibrosis and undertook this scanning electron microscopic study because methacrylate casts of capillaries are readily visualized by this instrument. We adapted the morphologic grid of Weibel[8] to scanning electron microscopy to obtain semiquan-

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titative, comparative data, similar to what we reported in the past.[9] We chose the bleomycin model, which has been employed in many studies, to produce the lung fibrosis.[10,11]

Materials and Methods

In a preliminary study, we decided to cast the vessels in a closed-chest animal model to better preserve the morphologic features. We cast the vessels at several inflation pressures. However, because vascular filling by the casting material was decreased even when a tracheal air pressure of only 2 cm H_2O was used to inflate the lungs, we chose to study the resting lung volume at the time of death of the animal.

Seventy female, Sprague–Dawley rats weighing between 150 and 250 g were anesthetized with 6 mg of pentobarbital sodium. The left and right ventricles were punctured to obtain blood for arterial and mixed venous gas analyses. Bleomycin (Blenoxane, Bristol), 1.5 or 2.4 units, in 0.25 ml of saline was instilled endotracheally into half the animals, and an equal volume of normal saline alone was instilled into the others.[12] After 30 days, the animals were again anesthetized; and arterial and mixed venous bloods were again sampled for oxygen, carbon dioxide, and pH. The abdomen was opened, and the inferior vena cava and aorta were cannulated below the level of the renal arteries. Heparinized saline warmed to 45 C was infused into the inferior vena cava at 50 cm water pressure until the aortic effluent was clear. Buffered 10% formaldehyde solution was then infused to fix the tissue. Twenty milliliters of methacrylate monomer (Mercox, Japan Vilene Co., Tokyo, Japan) mixed with catalyst was then rapidly injected into the inferior vena cava. Because polymerization began at once, the syringe pressure needed to deliver the material depended on the time after mixing with the catalyst. However, the pressure in the capillaries remained low, because no capillary rupture or extravasation of the casting plastic occurred. The animal was placed in a warm water bath for 1 hour to allow completion of the polymerization.

One lung alternating with the other was processed for either scanning electron microscopy or light microscopy. The paraffin sections were stained with hematoxylin and eosin (H&E) and elastic–van Gieson stain. With the light microscope, without the knowledge of which treatment the animals had received, two of us (N.W. and D.S.) together graded the extent of fibrosis, acute and chronic inflammation, and emphysema. A score of 1 was assigned for 0–5%, 2 for 6–25%, 3 for 26–50%, and 4 for greater than 50% involvement for each abnormality, similar to previously reported.[13] This subjective grading served to guard against untoward results such as no change from the bleomycin treatment and infection in both groups.

Table 1—Light-Microscopic Changes*

	Bleomycin	Saline	P
Fibrosis	2.50 (1.03)	1.04 (0.21)	0.0001
Chronic inflammation	2.13 (0.62)	1.30 (0.70)	0.0005
Acute inflammation	1.31 (0.60)	1.04 (0.20)	0.10

* One was scored for 0–5%, 2 for 6–25%, 3 for 26–50%, and 4 for 51% or greater involvement. The mean scores are given. The standard deviation is in parentheses, and the *P* values are from the Wilcoxon rank sum test.

The specimens to be studied by scanning electron microscopy were placed in concentrated NaOH solution until all the tissue was digested. The casts were rinsed in detergent, water, and ethanol, inspected with a dissecting microscope, and sectioned. Sections, about 1 mm thick, were fastened to the aluminum studs with silver conducting cement, sputter-coated with a layer of palladium gold about 20 nm thick, and viewed with a JEOL JSM-35C scanning electron microscope. The

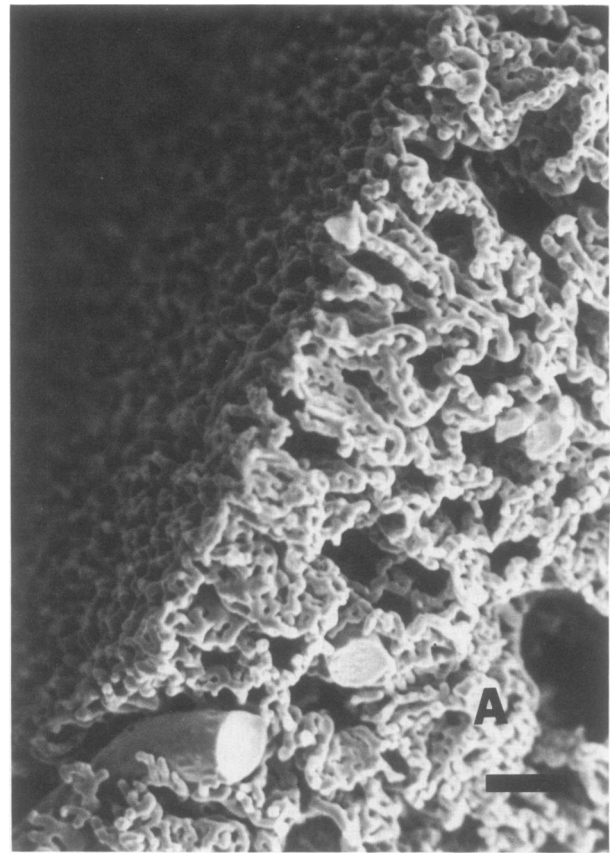

Figure 1—The alveolar surface (*A*) of this normal lung is seen *en face*, and the pleural surface (*P*) is to the top and left. The capillaries of the two surfaces are continuous. Bar is 50 μ. (×250)

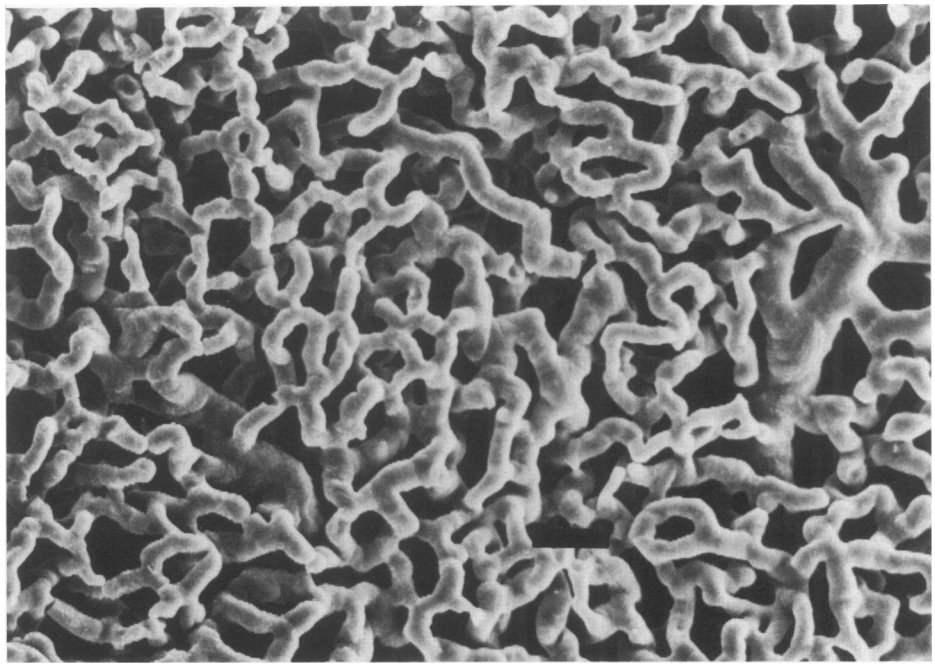

Figure 2—The pleural surface of this normal lung has tubular capillaries and larger vessels in two dimensional sheets. The capillaries have shorter distances from artery to vein and lack the complicated perialveolar basket structures that alveolar surfaces have. Bar is 20 μ. (×640)

final aperture was 200 μ; the accelerating voltage was 15 kv; and the working distance was 15 mm. Photographs for diameter and density measurements were taken at ×1000 magnification. The depth of focus was, therefore, 30 μ.[14] The areas on the specimen to be studied were selected at random, but a photograph was taken only if the region was adequately preserved. Additional micrographs were taken at lower magnification to trace capillary paths and to search for arteriovenous anastomoses. Sampling, grading, and counting were carried out by three observers (D.S., R.H., D.M.) together, who were unaware of the group from which the specimen came.

By scanning electron microscopy the casts of capillaries are easily identified because they are the smallest vascular structures, have no directionality and make up most of the vessels viewed. Capillaries are uniform in size, without tapering or change in diameter with branching. Branching usually occurs at about 90 degrees in T, Y, or psi shapes.[9] A four-point grid was superimposed on the micrograph, and the diameter of the closest capillary to each point was measured. Alveolar and pleural capillaries were analyzed separately. Up to four capillary diameters could be measured from each of 200 micrographs.

To estimate the relative density of the capillaries, we laid a 21-line, 42-point grid over the micrographs and used two counting methods. In the first, the points that overlay the capillaries and those that overlay the areas between capillaries in the plane of focus were counted. The number of points falling over the capillaries, divided by the number of points falling over and between capillaries, was taken as a measure of capillary density. Nonfilling, ambiguous, and uncertain points were ignored. The second counting method was similar, except that the points that did not land on a capillary were counted as misses even though they did not fall in an area that was bounded on all sides by capillaries. Again, nonfilling, ambiguous, and uncertain points were ignored.

On 42 vascular networks, we traced a capillary as far as possible, counting the number of branches it gave off. Regardless of how the dividing occurred, each twig was considered one branch. We traced the length of a circuit from its last arterial branch to the beginning of its vein, again noting the number of branches per distance. These branching frequencies were compared in the two groups by themselves and with respect to their luminal diameters.

Alveolar capillaries have been described to be arranged in polygonal rings.[8] The largest diameter of the center of these rings was recorded and number of spokes (capillary branches) attached to it were measured.

Parametric comparison was made by the *t* test, and nonparametric comparison was carried out by the Wilcoxon rank sum test. Normality was determined by the Kolomogorov D or Shapiro–Wilk statistic.[15]

Results

Although each group started with 35 animals, 9 died because of anesthesia, cardiac punctures, or the surgical procedures; so that the bleomycin group had 31 and the saline group 30 animals. Animals that received 1.5

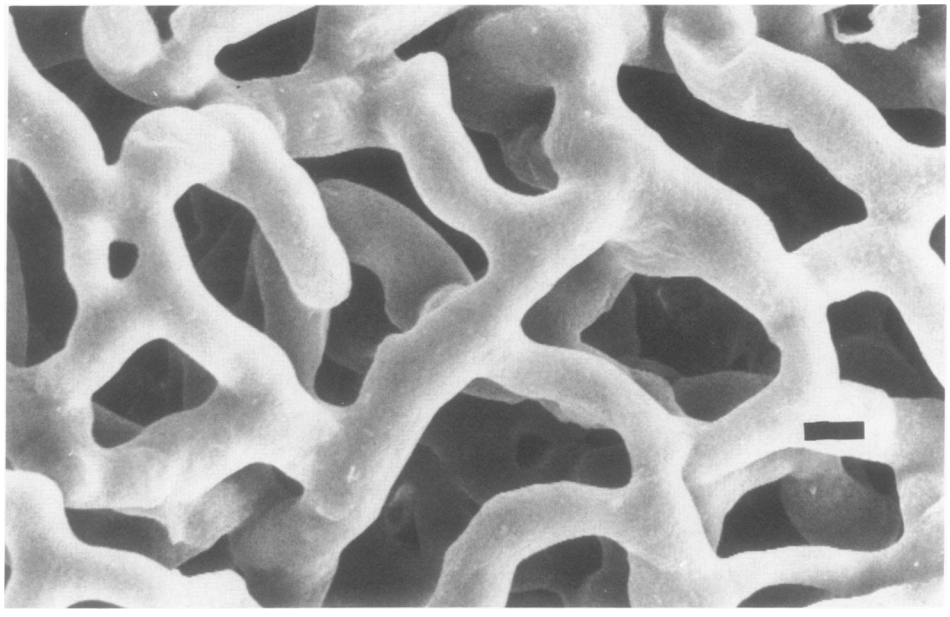

Figure 3—The pleural surface of this saline animal lung has loose capillary loops, wider intercapillary space, and less frequent branching than the alveolar capillaries. Bar is 5 μ. (×2000)

units of bleomycin were not distinguishable grossly or in any measured parameter from those receiving 2.4 units. Therefore, both were grouped together. Seven lungs appeared on gross examination to have diffuse mottling. Six of these were in the bleomycin group, and one was in the saline group. Pleural adhesions occurred in 1 animal in each group. In 1 animal in the bleomycin group, in which diffusely mottled fibrosis developed and which appeared to have some emphysema by gross examination, CO_2 retention developed. Shunting increased by 2% in the normal group and 7% in the bleomycin group, a difference that was not significant.

The light microscopy showed fibrosis and chronic inflammation in the bleomycin group, although the changes were patchy and varied in extent (Table 1). The fibrosis and chronic inflammation occurred mainly around the bronchi and bronchioles and near the pleura. A few lungs had extensive diffuse damage, but honeycombing was not found.

On scanning electron microscopy in normal lungs the pleural and alveolar capillaries were easily distinguished (Figure 1). The pleural capillaries were round or oval and loosely arranged in two-dimensional sheets (Figures 2 and 3). The alveolar capillaries often flattened as they approached the alveoli and molded to the alveolar spaces, forming basketlike structures previously described by Ohtani[16] (Figure 4). The peribronchial and periarterial capillaries had a sparser pattern similar to the pleural capillaries, but were arranged in single layers around the larger structures (Figure 5). One capillary often served more than one alveolus.[17] Indeed, often the entire blood supply of an alveolus was supplied by one capillary. We traced more than 30 branches within an alveolus without completing an arterial-to-venous circuit. The alveolar capillary bed arborized more than the pleural. We could not identify with certainty anastomoses of vessels larger than capillaries in either group. Small capillaries, less than 5 μ in diameter, were observed in the parenchyma of the normal group (Figure 6). Casts frequently ended blindly in the subpleural and pleural areas. Connections between pleural and pulmonary capillaries were extensive (Figure 1).

In the animals with fibrosis some distinctive features occurred in the alveolar capillaries, but many micrographs were not readily distinguishable from the normal, especially at low magnification (Figures 7 and 8).

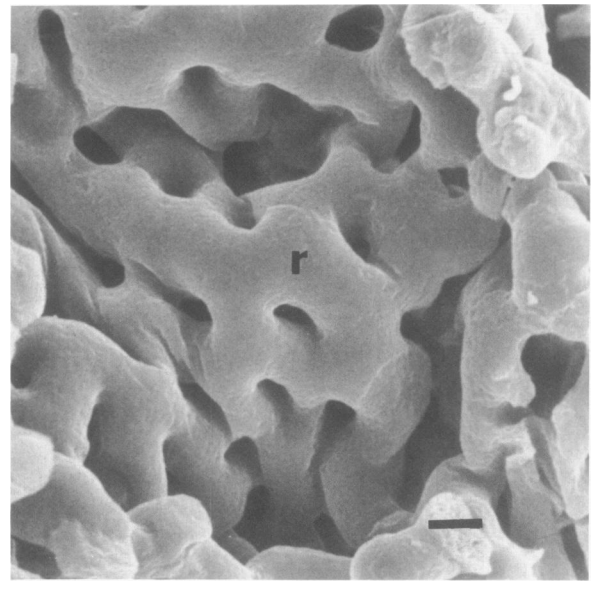

Figure 4—The alveolar capillaries of a normal animal show the ring structures (*r*) are square at lower lung volumes. Bar is 5 μ. (×2000)

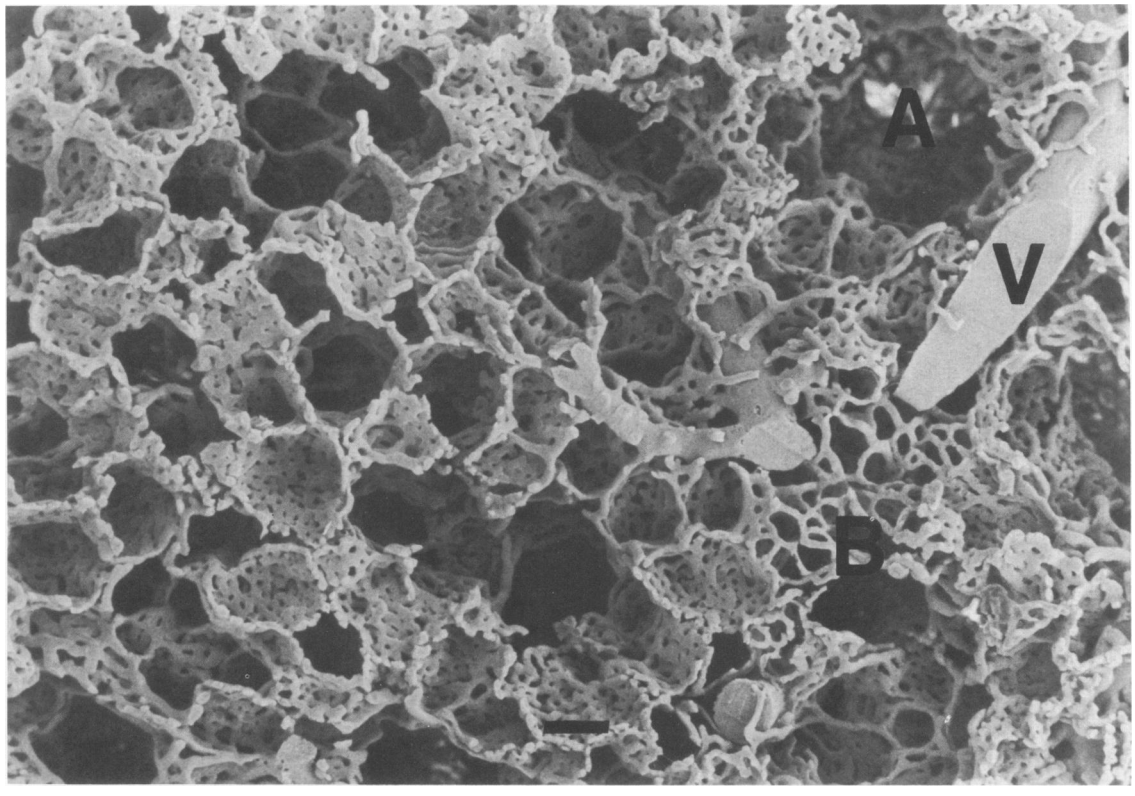

Figure 5—There is a large vessel (*V*) next to where an airway was (*A*). The bronchial capillaries (*B*) around these structures are loosely arranged like pleural capillaries. These capillaries are also continuous with the alveolar capillaries. Bleomycin-treated animal. Bar is 50 μ. (×175)

However, the intercapillary spaces appeared greater and the capillary rings were less regular (Figure 9). Irregular dilations of the capillaries occurred. Rarely, giant capillaries, up to 19 μ in diameter, were noted (Figures 10 and 11).

The capillary diameters were normally distributed in both the pleural and alveolar bed of the saline group and in the pleural, but not the alveolar, capillary bed of the bleomycin group ($P < 0.01$). In both the pleural and alveolar surfaces, the diameters of the capillaries

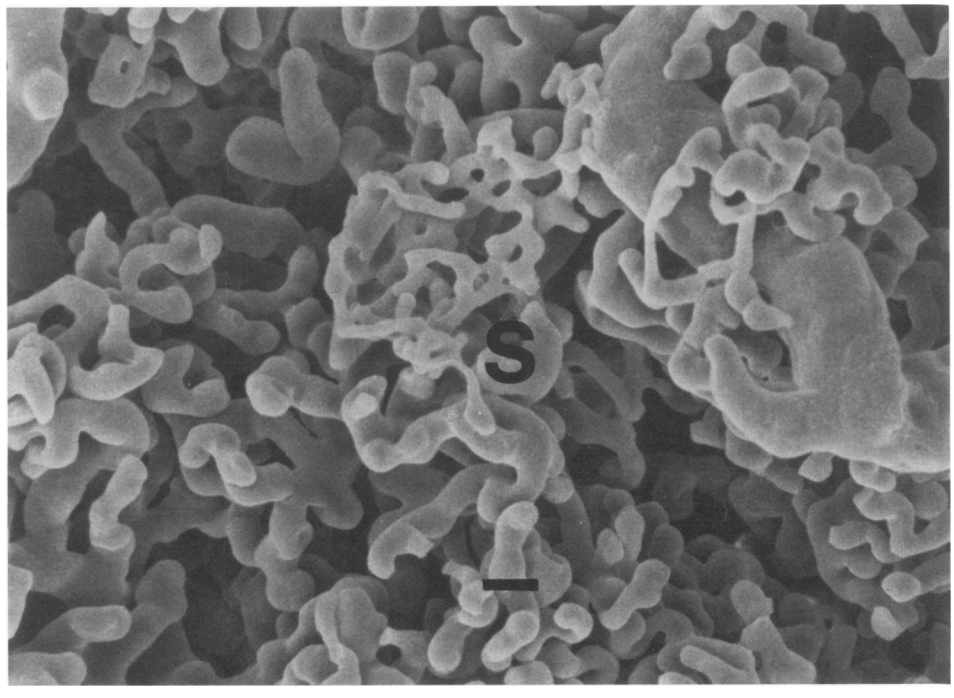

Figure 6—Capillaries too small (*S*) to allow passage of erythrocytes rarely occurred in both groups. Saline animal. Bar is 10 μ. (×720)

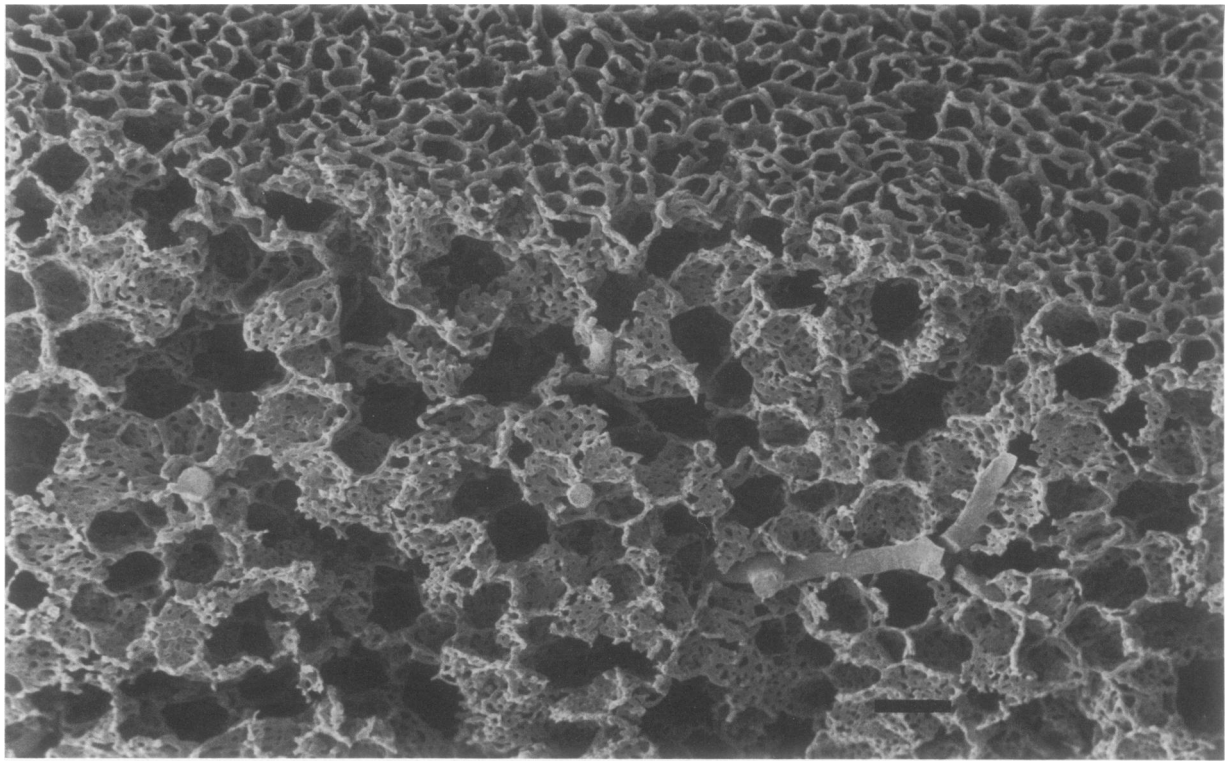

Figure 7—The top, pleural surface, has loosely arranged capillaries in sheets. The bottom is alveolar. Although this animal received bleomycin, the vessels seen here appear almost normal. Bar is 10 μ. (×100)

of the bleomycin group were larger than in the saline group ($P < 0.01$) (Table 2).

The densities measured by the first counting method were not different between the bleomycin and saline groups in either the alveolar or pleural surfaces, but the second counting method showed that the pleural surface of the bleomycin animals was significantly decreased (Table 3).

In normal animals the alveolar capillaries had 16 branches per 100 μ; in the bleomycin group they had

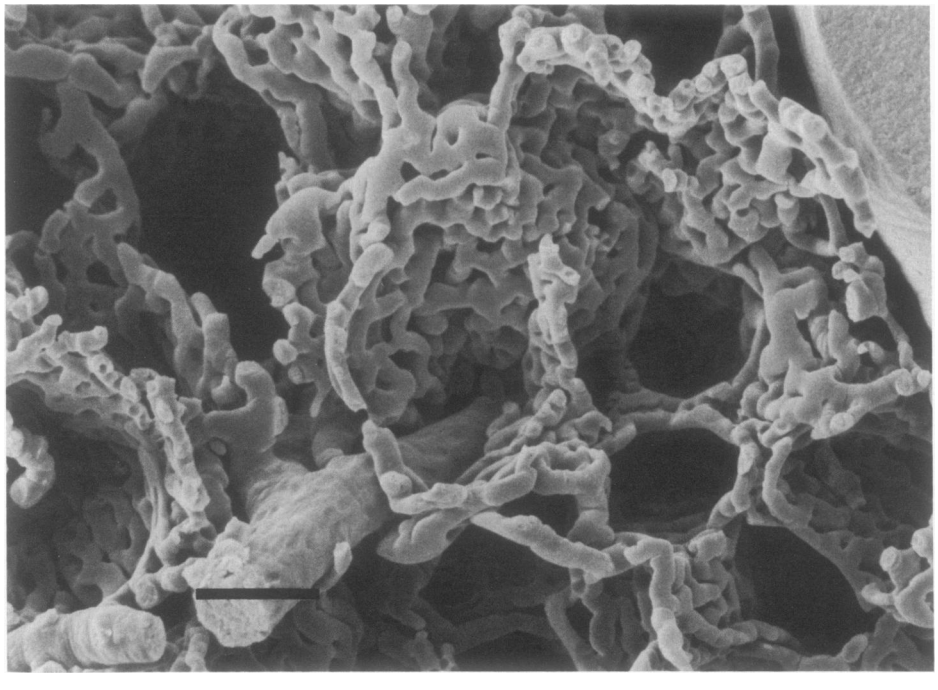

Figure 8—Alveolar capillaries from a bleomycin-treated animal appear almost normal. The polygonal rings are less regular, and the central holes are larger than normal animals. Bar is 5 μ. (×390)

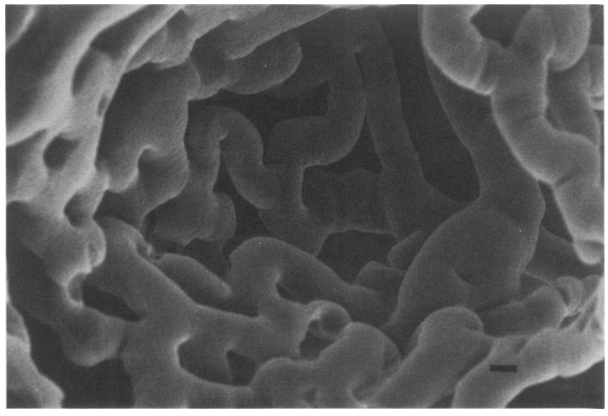

Figure 9—An alveolar basket in a bleomycin-treated animal. Branching is decreased. The area between capillaries is increased. Bar is 5 μ. (×1400)

11 branches (P = 0.02). This branching frequency meant that the alveolar capillaries in the saline group gave off a branch every 1.15 diameter's distance (or a dichotomous branch half as often), and the bleomycin group gave off a branch about every 1.3 diameter's distance (P = 0.06) (Table 4). Pleural capillary branching appeared unaltered by the fibrosis and occurred about every 1.6 diameters. Although we were generally unable to trace a complete arterial–venous course, one complete alveolar circuit that we were able to count had 28 branches. The short pleural circuits that we were able to trace had between 3 and 15 branches.

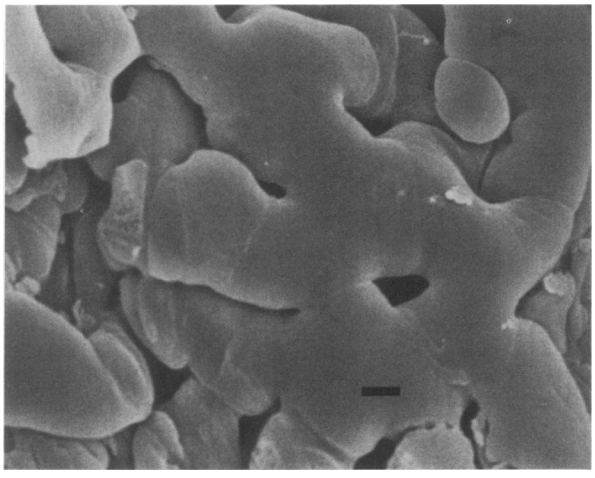

Figure 11—Alveolar capillary casts of a bleomycin-treated animal with large dilated capillaries. Bar is 5 μ. (×1520)

The diameters of the center of the alveolar capillary rings were greater in the bleomycin group (P = 0.03) (Table 5), but the number of branches that could be seen coming from each ring was not.

Discussion

Controversy exists over the interpretation of the normal lung capillary structure. Instead of a network of tubular structures, as in other organs, a sheet-flow

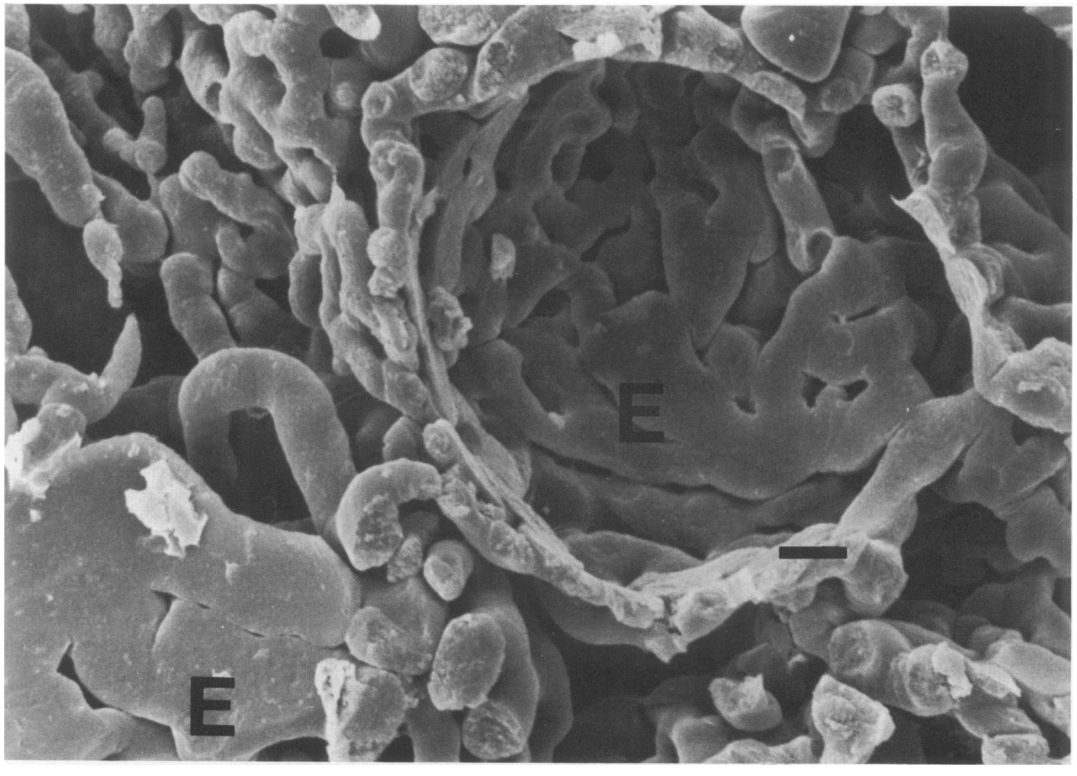

Figure 10—The capillaries in and around an alveolus of this bleomycin-treated animal have bulging enlargements (*E*). Such vascular lakes were not seen in normal animals. Scale is 10 μ. (×900)

Table 2—Diameters of Capillaries in Microns*

Surface	Bleomycin (n)	Saline (n)	P
Pleural	7.87 [1.6] (100)	7.23 [1.4] (96)	0.004
Alveolar	7.64 [2.3] (102)	6.75 [1.4] (100)	0.003
P	0.07	0.02	

* The number in each group is in parentheses and the standard deviation is in brackets. The P values are from the Wilcoxon rank sum test.

model has been suggested by Fung and Sobin.[18,19] Guntheroth and colleagues have disagreed and supported the tubular model.[20] We noted the compactness and slight widening of normal perialveolar capillaries as they abut against the alveoli. At low lung volume they have a grill- or sheet-like appearance. However, we found the capillaries to be tubular structures on the pleural surface and in most of the parenchyma at higher lung volume.

In both normal and fibrosis animals we found capillaries less than 5 μ in diameter. This is unlikely to be solely the result of shrinkage from fixation,[21] because these small capillaries were localized. Similar structures can be seen on a published micrograph of Hijiya, who cast the lungs of rats after giving them intraperiotoneal bleomycin sulfate.[22] In his model, endothelial swelling occurred and could have caused decreased capillary diameters. Endothelial swelling is an early occurrence in the lung injury caused by intraperitoneal bleomycin but is less noted in the intratracheal model. We cast the pulmonary circulation at functional residual capacity at death. Assimacopoulos and colleagues noted folding and decrease in capillary size at low lung volumes.[23] It is possible that these localized small capillaries resulted from the low lung volume at which casting was carried out. However, the mean capillary diameter in our normal animals was larger than[20] or about the same as[17] what others have reported. The differences between the diameters we found and those reported may result from our distinguishing between pleural and alveolar capillaries.

Table 3—Capillary Density*

Surface	Bleomycin	Saline	P
Counting Method 1†			
Pleural	0.65 [0.10] (16)	0.63 [0.07] (20)	NS
Alveolar	0.86 [0.07] (11)	0.90 [0.06] (7)	NS
Counting Method 2‡			
Pleural	0.77 [0.09] (91)	0.81 [0.08] (129)	0.01
Alveolar	0.85 [0.10] (100)	0.84 [0.08] (116)	NS

* The standard deviation is in brackets and the number of measurements is in parentheses.

† The ratio given is the number of points landing on capillaries (hits) divided by the number of points landing on capillaries (hits) plus the number of points landing on spaces wholly bounded by capillaries (misses).

‡ The ratio given here is the number of points landing on capillaries (hits) divided by the number of points landing on capillaries (hits) plus the number of points missing the capillaries (misses).

Table 4—Branching Frequency*

	Bleomycin (n = 12)	Saline (n = 12)	P
Alveolar surface			
Distance between branches			
In microns	9.97 (3.0)	7.08 (3.0)	0.02
In diameters	1.30 (0.4)	1.15 (0.4)	0.06
Branches			
Per 100 μ	11.01 [4.0]	15.90 [5.2]	0.02
Per diameter	0.84 [0.3]	1.07 [0.3]	0.06
Pleural surface			
Distance between branches			
In microns	8.84 (2.7)	9.89 (3.3)	NS
In diameters	0.70 (0.2)	0.69 (0.2)	NS
Branches			
Per 100 μ	12.62 (5.2)	11.60 (3.9)	NS
Per diameter	1.61 (0.7)	1.61 (0.5)	NS

* The standard deviation is in parentheses and the P values are from the Wilcoxon rank sum test.

Measurement of density in scanning electron microscopy is not yet established. Because of the great depth of field, it is necessary to restrict what is counted. Of the two counting methods of estimating density that we used, we favor the more restrictive (first) method. The number of points not counted as either a hit or a miss was greater in the first method (the saline group averaged 10 and the bleomycin group 5) than in the second method (the saline group averaged 3 and the bleomycin group 2). With the second counting method, the capillary density of the pleural surface in the fibrosis animals was decreased. The increased interstitial tissue that grows between alveoli in fibrosis causes the capillaries to lose their sheetlike compactness and decreases the parenchymal capillary density. However, the increase in capillary diameter causes an increase in density, which may account for our inability to find a difference in the alveolar capillary density between the normal and bleomycin-treated animals. We are planning to use computer-assisted image analysis to measure capillary density in a more reproducible and standardized form.

Table 5—Alveolar Capillary Ring Structure*

	Bleomycin (n = 27)	Saline (n = 26)	P
Hole size (μ)	5.30 (2.0)	4.23 (1.2)	0.03
Branches connecting to the holes	4.96 (1.4)	4.85 (0.7)	NS

* The standard deviation is in parentheses and the P values are from the Wilcoxon rank sum test.

Although the increased diameter of both the pleural and alveolar capillaries in fibrosis could be related to chronic increased blood flow or tethering of capillaries by retracting interstitial tissue, the configuration of the dilated capillary segments suggests that a disruption and remodeling occurred. The abnormal enlargements may be similar to the dilated capillary, cirsoid nests which Tomashefski and colleagues described in humans, dying after a prolonged course of adult respiratory distress syndrome.[24] It is also possible that the capillary bulged from the casting material, because the walls of recently remodeled capillaries may be weak and might bow with the pressure of the methacrylate being infused. However, this is unlikely, because the intracapillary pressure was so low that even slight alveolar pressure markedly decreased or prevented filling. Furthermore, the lungs were fixed with formalin before casting. Filling pressures cannot be accurately monitored during casting, because the viscosity of the methacrylate constantly changes, and the pressure away from the syringe rapidly decreases with intravascular branching and distance.

Even though there was an increase in the size of capillaries in the fibrosis animals, the area occupied by the alveolar capillaries was not increased, which means that there must be fewer capillaries. This agrees with the findings of Gracey and colleagues,[5] who noted a decrease in the number of capillaries in fibrosis. The branching decrease in fibrosis also suggests loss of the capillary bed. Our branching count is no doubt an understatement, because if an offshoot arose directly opposite the electron collector (camera), it could be missed. Similarly, a nonfilling limb could go unnoticed.

Furthermore, if there are fewer and larger capillaries, some must have disappeared and others must have increased in size. If the volume of the total capillary bed remained constant but the diameter of individual capillaries increased, then there would be a decrease in pulmonary capillary surface area. Oxygen transfer, which is dependent on the alveolar–capillary surface area, would be expected to be decreased; and this may partially explain the ventilation perfusion abnormalities seen in fibrosis.

The number of spokes coming from the polygonal ring structures of the alveolar capillaries does not appear to be altered, but we found the central hole size to be increased in bleomycin-induced fibrosis. The centers of these rings, or "holes," were originally thought to be the sites of Type II cells by Alexander and colleagues.[25] Hijiya and Okada also identified Type II cells and brush cells in the center of these rings. They further noted the ring was a square when the lung was fixed at low lung volumes,[26] a finding we also noted (Figure 4). These holes were later identified as the sites of the "pillars" by Fung and Sobin in their sheet-flow model, who found their dimensions varied with the thickness of the capillary sheets.[18,19] More recently, Assimacopoulos and colleagues have identified them as sites of contractile interstitial cells.[23] Their attractive hypothesis is that local blood flow is controlled by the contraction of these cells. They have shown that the cells contract under hypoxic conditions,[27] which alter the capillary configuration and, therefore, blood flow. Adler and colleagues have recently shown that these contractile interstitial cells are markedly increased in the lungs of rats with bleomycin-induced fibrosis.[28] If these holes are sites of interstitial contractile cells, and these cells are increased in fibrosis, then the increase in the size of the holes could be explained.

In summary, the appearance of irregularly shaped capillaries, an increase in diameter, and a decrease in branching are the main architectural changes of the pulmonary capillaries in lung fibrosis. Since there was no change in capillary density, a loss in capillary surface area must occur.

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