SCANNING ELECTRON MICROSCOPY OF THE DEEP LYMPHATIC NETWORK OF THE MURINE LUNG AS VIEWED IN CORROSION CASTS

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ABSTRACT

The lymphatic microvessels of the deep lung tissue were studied in corrosion casts, which were observed by scanning electron microscopy (SEM) after injection of a methacrylate resin (Mercox) through the trachea of CD-1 mice. We found that the deep lymphatics of the murine lung were composed of two interconnecting networks: a poorly developed capillary system located at the interacinar region, and a rich plexiform complex surrounding the bronchus. Lymphatic capillaries did not penetrate the alveolar area, thus leaving most of the lung parenchyma devoid of direct access to lymphatic drainage. Lung lymphatic vessels showed a small luminal surface and a low density of endothelial nuclei. Pulmonary lymphatic capillaries often formed star-like anastomoses. The structural features of pulmonary lymphatics, including their three-dimensional organization, were distinctly separate from those of the blood microvasculature of the lung.

The lymphatic system of the mammalian lung is usually divided into two compartments: (a) a superficial network located beneath the visceral pleura; and (b) a deep network within the lung parenchyma (1-5). Some lymphatic vessels link the two networks (1).

Whereas the superficial network is easily demonstrated by injection of markers into subpleural lymphatic vessels (e.g., barium sulphate) (1,5), a complete understanding of the deep lymphatic anatomy is still lacking. For instance, the relation between the deep lymphatics and the alveolar and bronchiolar walls remains unclear (6). Accordingly, we examined the lymphatics of the deep pulmonary tissue in mice after injection of a methacrylate resin (Mercox) through the trachea and bronchoalveolar space. Using scanning electron microscopy (SEM), we demonstrated a tridimensional arrangement and microanatomical features of the deep pulmonary lymphatics. The deep network of lung lymphatics consisted of two separate components: one made up of few lymphatics with an interacinar location and the other forming a rich lymphatic plexus in the peribronchiolar region (7).

MATERIALS AND METHODS

Animals

Female CD-1 mice, 6-8 weeks old, weighing about 20g were obtained from the

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animal house of the Gulbenkian Institute of Science (Oeiras, Portugal). The mice were housed in groups of 15 and given balanced food and water *ad libitum*.

Experimental Protocol

Five mice were anesthetized with diethylether, the trachea was punctured and a cannula was inserted and kept in place by a ligature. A methacrylate resin (Mercox® Cl-2R or Cl-2B of red and blue color, respectively; Japan Vilene Company, Tokyo, Japan) was infused into the trachea until the periphery of the lung turned either red or blue. Another group of five mice was used for blood vascular casting. Fifteen minutes before sacrifice, these mice were injected intravenously with 0.2ml heparin (1000 units). The mice were then anesthetized, the thorax was opened, the right ventricle was punctured, a cannula was inserted and immobilized by ligature. The aorta was cut to exsanguinate the mice and the vascular system was washed with heparinized phosphate buffered saline (37°C, 250 units heparin/10ml). Before the resin injection a perfusion fixation was made with an aldehyde mixture of 4% formaldehyde, 1.25% glutaraldehyde, and 10mM CaCl$_2$ in 0.05M cacodylate buffer. The vascular casting was done by hand injection of the resin using the cannula that had been previously inserted into the right ventricle. Twenty minutes later the resin perfused mice were warmed up to 25°C for 6-8 hours in order to obtain polymerization of the resin. To obtain the corrosion casts, the pulmonary biological material was digested using 25% sodium hydroxide at 30°C for 3-4 days. The resultant resin casts were cleaned by washing in distilled water with sonication and sectioned into 5mm thick slices to be studied by SEM.

In order to confirm that these casted vessels corresponded to lymphatics, we also compared the microvascular anatomy with that of lung blood vessels replicated by intracardiac injection of methacrylate resin.

For this purpose we used the additional mice that were perfused with the resin through the heart right ventricle.

Scanning Electron Microscopy

The corrosion casts were coated by Au/Pt under vacuum and examined in a JEOL JSM-35C scanning electron microscope. The electron micrographs were derived from secondary electron imaging (SEI) mode. The instrumental conditions for the observations were 15kV, 0.2nA, 30s/frame (photography). SEI was used for observations with an Everhardt-Thornley detector (8).

RESULTS

This technique yielded replicas of the lumen of lymphatic vessels associated with deep lung murine tissue. *Fig. 1A* shows a scanning electron micrograph of such a preparation. Lymphatic capillaries are seen on a background of multiple alveoli and it is clear that the lymphatic capillaries do not penetrate the alveolar domain; i.e., they stop before reaching the alveolar area of the lung (*Fig. 1B*). These lymphatics have a smooth luminal surface and follow relatively straight lines with bifurcating branches and curved arches (*Fig. 1A*). The lymphatic capillaries had irregular diameters that ranged in size from 10 to 50µ (*Fig. 2A*). The anastomoses of different lymphatic capillaries often formed star-like arrangements (*Fig. 1B*). The interacinar end of the lymphatic capillaries appeared to emanate from formations that resembled finger-like outpocketings (*Fig. 1B*, arrowhead).

In some preparations, valves were identified by the appearance of deep V-shaped imprints on the replicas (*Fig. 2B*). The nuclei of endothelial cells induced deep, rough, oval-shaped imprints in the casts; they were sparsely distributed along the capillary membrane (*Fig. 2A and 2C*). Adjacent to and encircling the bronchus, the lymphatics formed a well-developed plexiform layer (*Fig. 3*). This arrangement was distinct from
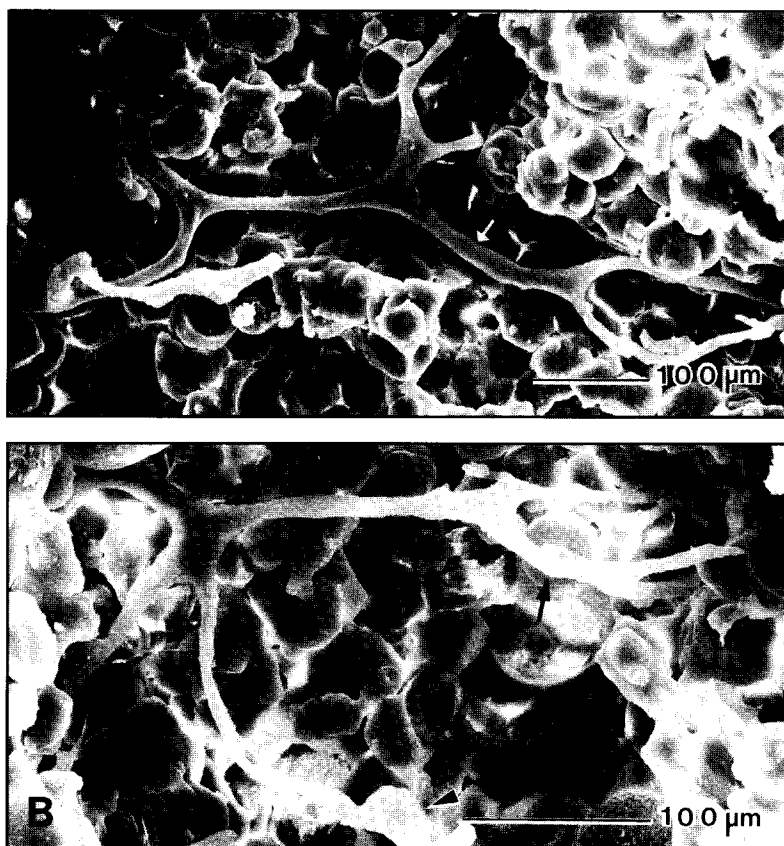
Fig. 1. Low magnification scanning electron micrographs of corrosion casts of mouse lung instilled with methylmethacrylate resin through the trachea. Both the alveolar sacs (background) and lymphatic microvessels (arrows in foreground) were permeated by the resin. The lymphatics do not extend into the alveolar domain and have a bifurcating pattern (Fig. 1A) or form a star-like arrangement (Fig. 1B). In Fig. 1B a finger-like outpocketing appears as the beginning of a lymphatic capillary (arrowhead). Ax150, Bx300.

that of lymphatics near the interacinar region (vide supra) (compare *Fig. 3* with *Figs. 1A and 1B*). In fact, the plexiform peribronchial lymphatics formed a dense mesh of lymph vessels that sharply contrasted with the scarce density of interacinar lymphatics.

As shown in *Figs. 4A and 4B*, the SEM structure of the casted lung blood microvasculature was clearly distinguishable from that of the lymphatic vessels, with the density of pulmonary blood capillaries much greater than that of lymphatic microvessels. Blood microvessels also showed rougher luminal surfaces, a higher density of endothelial nuclei and a more complex branching pattern compared with lymphatics.

DISCUSSION

We demonstrated here the three-dimensional architecture of the lymphatic vessels of the deep lung tissue using SEM of Mercox resin corrosion casts. Lymphatic vessels were relatively scarce near the alveolus (often forming a star-like anastomotic pattern), whereas lymphatics adjacent and encircling the bronchus were more dense.

To our knowledge, this is the first study that illustrates the deep network of lung lymphatics using SEM. Previous investigations were restricted to either light or transmission electron microscopy (9,10). These reports suggested that the deep lung tissue

Fig. 2. Scanning electron micrographs of interacinar lymphatic vessels of the mouse lung showing different diameters of the same lymphatic microvessel (Fig. 1A, compare the diameters highlighted by arrowheads), the presence of V-shaped valvular imprints (Fig. 2B, arrowhead) and the sparsity of the nuclear imprints on the lymphatic wall (Fig. 2C, arrowheads). Ax470, Bx1200, Cx1000.
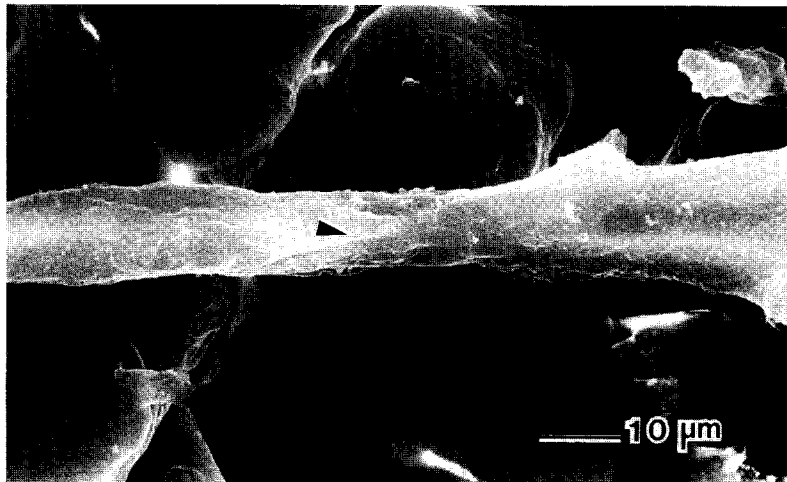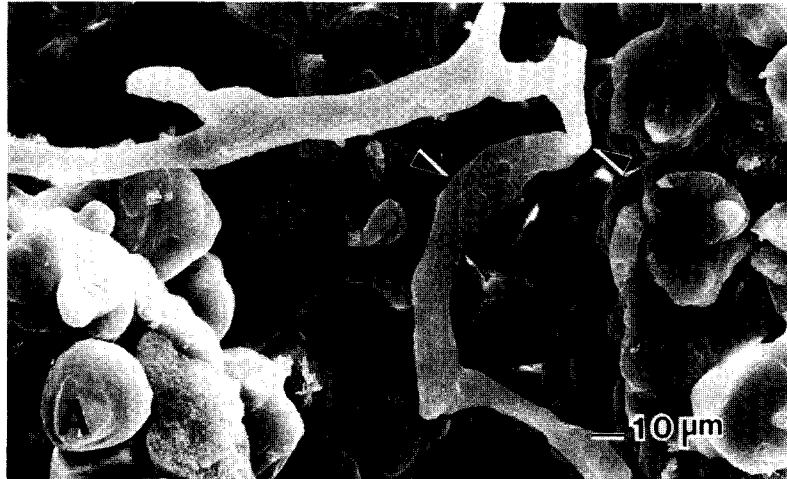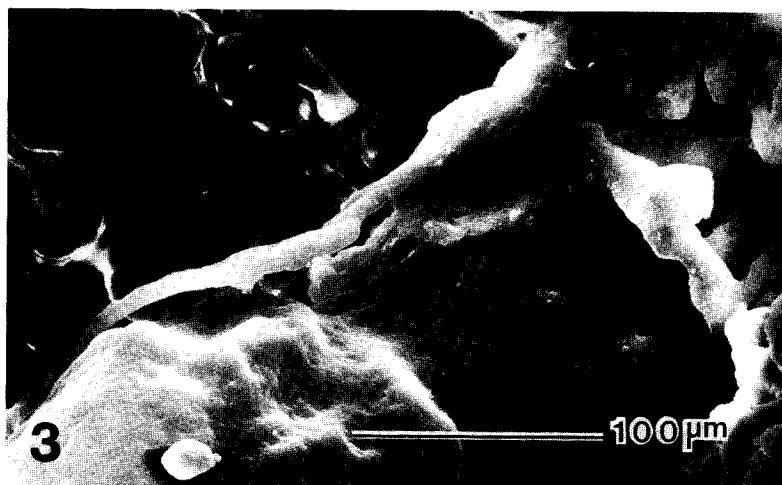
Fig. 3. Scanning electron micrograph of lung lymphatic vessels forming a plexiform arrangement around a bronchus, x390.

and in particular the alveolar region had a paucity of lymphatic capillaries (8,9,11). Our tridimensional views using SEM conforms with this view.

To corroborate the lymphatic origin of the casted microvessels, we compared the fine structure of the casts with that of replicas of blood vessels of the lung made with the same resin (12). In fact, SEM of vascular casts is an excellent method to distinguish between lymphatic and blood capillaries using the microanatomical features that separate these two microvasculatures (13,14). The SEM features of the presumed lymphatics are clearly different form blood microvessels and moreover are typical of lymphatics with smoothness of the microvascular wall, low density of endothelial nuclei, absence of sphincteric imprints and the presence of V-shaped valvular imprints (13).

We describe new topographical features of lung lymphatics, namely the formation of star-like anastomoses of capillaries, and the plexiform arrangement of the lymphatic microvessels that surround the bronchial tree. We confirm that lymphatics begin at the interacinar region, and that the respiratory septum is devoid of direct lymphatic drainage (1,9,15). The lack of direct lymphatic pathways probably explains the slow migration

rate of particle-containing macrophages from the alveolus to regional lymph nodes (16,17).

Although the lumen of lymphatics of the deep lung tissue were permeated after resin injection through the bronchoalveolar space, under physiological conditions, the broncho-alveolar space may not have direct continuity with the lymphatic system. Perhaps filling of the lymphatics was the result of creating artifactual communications between the two spaces by forceful instillation of the resin. On the other hand, filling of the lymphatic micro-vessels by the resin was not associated with rupture of the alveoli in the corrosion casts. It is also possible that tiny lymphatic fenestrae exist near the bronchiolar wall. If this latter structural concept is verified, then lymphatics may play a direct role in the clearance of particulates and macromolecules from the intrapulmonary airway sector of the lung.

Note added in proof. After this work was submitted for publication, an article on lung lymphatics by D.E. Scraufnagel appeared in print (*Anat. Rec.* 1992, 233:547-554). The general conclusions of this paper are consistent with our data; the results herein extend the microanatomical information on the lymphatics of the peribronchial and the interacinar regions of the lung.

Fig. 4. Scanning electron micrographs of casted lung blood vessels. Fig. 4A shows blood microvessels of an interstitial (connective tissue) area of the deep lung and Fig. 4B demonstrates alveolar blood capillaries; note the extensive arrangement of the blood capillaries forming a dense basket-shaped meshwork. Ax1600, Bx1000.
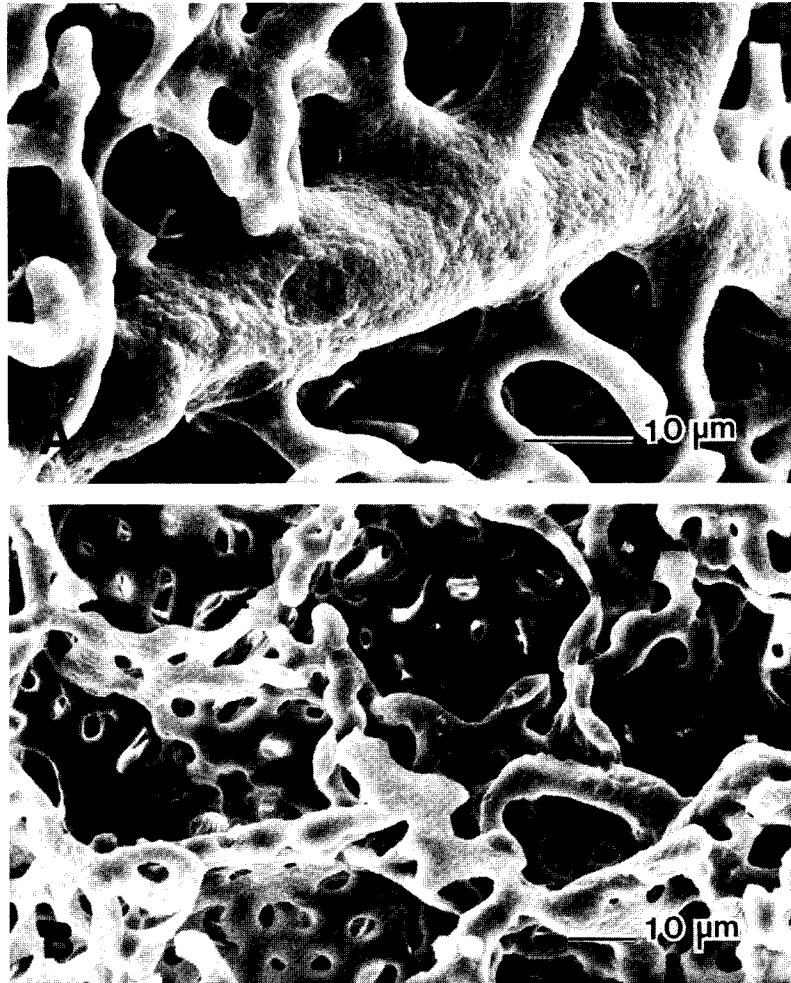
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