Kinetics of fluid flux in the rat diaphragmatic submesothelial lymphatic lacunae

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MATERIALS AND METHODS

Animal handling and surgical procedures. Adult male Wistar rats (mean body weight: 400 ± 18 g, n = 7) were housed and handled following the guidelines of the University of Insubria ethical research committee by which all protocols were approved. Rats were anaesthetised with an intraperitoneal injection of 75 mg/kg ketamine (Sigma Aldrich, Milan, Italy) plus 0.5 mg/kg medetomidine (Domitor, Pfizer). Successive half-boluses of the same anesthesia cocktail were administered every hour until the end of the experiments, when animals were euthanized via an anesthesia overdose. Before the surgical procedure was started and at regular time intervals during the experiment, the attainment of a deep surgical anesthesia level was assessed on the basis of the lack of the corneal reflex. Once deeply anaesthetised, the rats were turned supine on a warmed (37°C) blanket; they were then tracheotomized, and a T-shaped cannula was inserted into the trachea and connected to a heated pneumotacograph (model 8420, Hans Rudolph) equipped with a dedicated pneumotach amplifier (model 1110A, Hans Rudolph) to continuously record respiratory flow. The flow signal was then digitized with a National Instruments BNC-2090 analog-to-digital board (sampling rate: 100 Hz) and integrated through dedicated LabView software (National Instruments) to obtain respiratory tidal volume; both flow and volume signals were then displayed on a monitor.

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The femoral vein was exposed, and animals were paralyzed with a single bolus of 0.2 ml pancuronium bromide (Sigma Aldrich) solution (2 mg/ml in saline) administered in the vein through a 27-gauge needle. Immediately after paralysis, the tracheal cannula was connected to a mechanical ventilator (Ugo Basile, Como, Italy), and rats were ventilated in air with a tidal volume of 4 ml and a frequency of 60 breaths/min. At the onset of mechanical ventilation, the chest was opened, and the more caudal four to five ribs were bilaterally removed to expose the whole diaphragm, with care taken not to pierce the diaphragmatic surface and/or proximal abdominal wall.

The diaphragmatic lymphatic network below the pleural surface of the diaphragm (Fig. 1A) was explored with the aid of a stereomicroscope (SV11 Carl Zeiss, Milan, Italy). The diaphragmatic surface was frequently rinsed with a gentle flush of saline to avoid dehydration.

Lymph flow velocity. With the use of a vertical puller (PP30, Narishige, Tokyo, Japan), glass pipettes for microinjection were pulled from borosilicate glass capillaries (1B100-4, 1.0 mm outer diameter, 0.58 mm inner diameter, WPI Europe, Berlin, Germany) to tip diameters of ~20 μm. Pipettes were filled with mineral oil and mounted onto a mechanical microinjector (WPI Europe) set to deliver 4.6 nl/injection. Under the stereomicroscope, pipettes were front filled with 1 μl of 0.2 μm filtered 2% FITC-dextran (282 kDa)-conjugated solution in saline. With the use of a mechanical coarse/fine micromanipulator (Narishige), the pipette was longitudinally aligned over the lymphatic vessel to be injected with the shallowest possible angle with respect to the diaphragmatic surface. Careful positioning of the pipette tip with respect to the lymphatic vessel proved to be a key point for an almost-complete injection success rate into the vessel itself. After being positioned, the pipette tip was gently advanced through the pleural diaphragmatic surface until it was clearly inside the lymphatic vessel lumen. A single injection of 4.6 nl volume was then triggered, and the pipette tip was immediately withdrawn from the vessel.

All phases of pipette positioning, fluorescent dye injection, and distribution of the dye into the lymphatic vessel lumen were recorded. The stereomicroscope was equipped with a cooled black-and-white charge-coupled device camera (ORCA ER, Hamamatsu, Milan, Italy) connected with a personal computer running SimplePCI software (Hamamatsu) to control all the aspects of image acquisition and analysis. FITC-dextran conjugate was excited with an optic fiber epi-illuminator equipped with a 425 ± 65-nm bandpass filter (Carl Zeiss Italy). Fluorescence emission was collected by the stereomicroscope fitted with a two-bandpass filter (FITC/TRITC, catalog no. 56918, Chroma Technology, Rockingham, VT). Images were collected both in real time, to better follow the time course of the injection, and in time lapse (interframe interval: 10–30 s) a few minutes after the injection. The exposure time was routinely set to 0.3 s or lower to allow the detection of the faintest fluorescence signals without a significant increase in background noise. Images were directly stored in the hard disk of the computer and subsequently analyzed offline. Typical recordings of a single-vessel injection did not last >25 min. To obtain the most clear and still images of lymphatic vessels during time-lapse recording, the diaphragm was temporarily (~2–3 s) arrested by occluding the inspiratory line of the mechanical ventilator during image exposure and immediately reopening the inspiratory line after image acquisition. During real-time recording, the inspiratory line was kept closed for time periods no longer than 10 s during a period of no more than 3 min, allowing several breaths in between. For each vessel, several measurements were made in each segment.

Direct measurements of vessel diameter and fluorescence progression velocity were performed on original images with SimplePCI software. Flow velocity was computed by dividing the distance traveled by the fluorescence front along the path of the lymphatic vessel (Fig. 2, A and B) by the interpicture time interval.

Recording of intraluminal lymphatic pressure. One cannot exclude that the measured lymph flow velocity might have been affected by changes in intraluminal lymphatic pressure (Plymph) induced by the injection maneuver. To evaluate the occurrence of this possible artifact, P_{lymph} was measured during and after the injection maneuver with the use of the micropuncture technique. P_{lymph} recordings were attained through glass micropipettes bevelled down to 2- to 4-μm tip diameter, filled with 1 M NaCl solution, and secured to a pipette holder filled with the same solution. The holder was connected to a mineral oil-filled pressure transducer (Gould Instruments System), which was motor driven by an electrohydraulic system (Dual Servomull Pressure-Measuring System, Vista Electronics, Ramona, CA). The pressure signal was relayed to an amplifier and a signal conditioner (model 6600, Gould Electronics), digitized with an analog-to-digital board, and displayed on the monitor using dedicated LabView software (National Instruments). Before being used, each micropipette

Fig. 1. A: stereomicroscope image of a linear lymphatic vessel under white light epi-illumination. Lymphatic vessels appeared as darker-than-background conduits delimited by faint white borders. Smaller tributary vessels were seen as vessels emptying into the principal, slightly curved vessel. Scale bar = 100 μm. B: stereomicroscope image of a lymphatic loop on the pleural surface of the diaphragm under white light epi-illumination. From a single linear vessel, the loop branched into shorter ducts placed in parallel and/or in series with each other. Therefore, at variance with the linear vessel shown in A, the loop offered multiple paths for fluid recirculation. Underneath the lymphatic loop, the diaphragmatic muscular fibers were distinguished and crossed the loop almost perpendicularly. Scale bar = 200 μm. C: a known volume of mineral oil injected through a micropipette filled a linear vessel segment, allowing the derivation of vessel thickness. See METHODS for further details. Scale bar = 100 μm.
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was calibrated in a modified Lucite box by imposing step changes of ±5 mmHg in the box chamber; pipettes displaying a nonlinear calibration in the pressure range of ±30 mmHg were discarded. After calibration, pipette holders were mounted in two three-dimensional hydraulic micromanipulators (Joystick Micromanipulator MO-188 or MO-109, Narishige) equipped with a fourth micromanipulator movement to drive the tip of the micropipette into the tissue. Electrical zeroing of the recording system was performed before and immediately after each measurement by dipping the micropipette tip in a saline pool positioned at the same height of the pipette insertion point.

Criteria for acceptance of the micropipette pressure recordings were: 1) an unchanged electrical zero of the system on withdrawal from the tissue compared with the preinsertion value, 2) a stable pressure reading for at least 2 min, and 3) repeated measurements from the same area were within ±1 mmHg.

Estimates of lymphatic vessel thickness and lymph flow. Lymph flow (V_{lymph}) was calculated as the product of lymph flow velocity (v) by vessel cross-sectional area (S) as follows:

\[
V_{\text{lymph}} = v \times S = v \times \left( \pi \times \frac{d}{2} \times \frac{t}{2} \right)
\]

(1)

where d and t are the diameter of the vessel on the diaphragmatic plane and on the axis perpendicular to the diaphragmatic surface, respectively. For simplicity, we referred to t as vessel thickness. Parameter d was directly measured on recorded stereomicroscope images of the vessel through SimplePCI software, whereas t could be only indirectly measured in three of the rats, which had undergone the same surgical preparation to expose the diaphragm as previously described. Under stereomicroscopic view and with the aid of a pipette micromanipulator, a bolus of mineral oil of known volume [injected oil volume (V_{inj}): 2.6 nl] was injected in a linear vessel or a lymphatic loop branch while images were recorded by the cooled black-and-white charge-coupled device camera before the injection and throughout the whole injection maneuver. In contrast to the behavior of the fluorescent dye dispersed in saline, the oil 1) did not mix with the fluid present in the lymphatic vessel before the injection; 2) did not rapidly flow into the vessel away from the injection point but remained confined in a single, well-defined droplet;}

\[
V_{\text{inj}} = \left( \frac{d_{\text{inj}}}{2} \times t_{\text{inj}} \right) \times \frac{4 \times V_{\text{inj}}}{\pi 	imes A_{\text{inj}}} 
\]

(2)

where \( t_{\text{inj}} \) is the postinjection vessel diameter perpendicular to the diaphragmatic surface. On bidimensional images, such as that shown in Fig. 1C, the oil drop filled an almost rectangular area, so that one may approximate \( l \) as follows: \( l = A_{\text{inj}} / d_{\text{inj}} \). Hence, \( t_{\text{inj}} \) can be calculated from Eq. 2 as follows:

**RESULTS**

On the diaphragmatic surface, noncounterstained subpleural lymphatic vessels could be distinguished from the surrounding tissue, blood vessels, and nerves based on their distinctive appearance under semitangential epi-illumination, with a darker-than-surrounding lumen lined by whitish translucent borders (Fig. 1, A and B). Lymphatic vessels could be grouped into two populations depending on whether they ran in a more or less tortuous, but approximately linear, path (Fig. 1A) or were organized into loops (Fig. 1B). The latter branched from a single linear vessel, bifurcated into two to five shorter ducts placed in parallel and/or in series with each other, and then emptied into a linear vessel running toward the diaphragmatic phrenic center. Therefore, at variance with linear vessels, loops offered multiple paths for fluid recirculation. Notwithstanding the great length variability, linear vessels were significantly (P < 0.05) longer (8,106.5 ± 3,137.7 μm, \( n = 18 \)) than vessels arranged in loops (2,492.9 ± 1,954 μm, \( n = 21 \)). The latter were preferentially located in the peripheral ventrolateral regions of the diaphragm, whereas linear lymphatics were dispersed over the whole surface, apparently connecting different regions. No loops were detected in the medial tendineous portion of the diaphragm.

Diameters of the two populations of lymphatic vessels were normally distributed and significantly greater (P < 0.01) in linear vessels (103.4 ± 8.5 μm, \( n = 18 \), range: 54.6–170.5 μm) than in loops (54.6 ± 3.3 μm, \( n = 21 \), range: 38.3–98.1 μm), which also appeared to be more uniform in size. In the set of lymphatic vessels used for the evaluation of vessel thickness, postinjection thickness averaged 48.8 ± 4.7 μm (\( n = 16 \)), with no differences between linear vessels and loops. However, as shown in Fig. 1C, the injection locally deformed the vessels, with the ratio between pre- and postinjection diameter being 0.7 ± 0.05. Assuming a similar ratio in thicknesses, the average preinjection thickness would be 36.1 ± 6 μm.
Flow velocity was computed on frames like those shown in Fig. 2, which shows two subsequent frames from a set of images of a linear vessel taken after intraluminal injection of fluorescent dextran. In the frame shown in Fig. 2B, which was taken 28.2 s after the frame shown in Fig. 2A, the fluorescent dye front, detected by the thin horizontal line, progressed by 627.3 μm at a velocity of 22.2 μm/s, which was calculated by dividing the distance traveled along the path of the vessel by the time interval. The spreading velocity of the fluorescent dye within the vessel may be considered as a reliable tool to derive the intraluminal flow velocity, provided that the probe freely moves within the vessel without escaping from the surface, at least during the observation frames. To verify the adequacy of the latter assumption, we tested whether FITC-dextran could leak out of the vessel in a time span of 30 min, longer than our longest actual recording time of ~22 min. Thirty minutes after FITC-dextran injection, the fluorescence signal was entirely confined to the lumen (Fig. 3A). Although one cannot exclude that a subtle amount of leakage below the detection limit of our system might have occurred, no sign of FITC-dextran leakage was observed, even after image processing to amplify the fluorescence signal (Fig. 3A, inset).

As shown in the example in Fig. 3B, immediately after the injection, \( P_{\text{mean}} \) recorded in close proximity to the injection pipette tip increased from a baseline value of 1.5 ± 0.4 mmHg to 5.6 ± 2.8 mmHg \((n = 7)\), thereafter declining back to baseline with a time course well fitted by a double-exponential decay with a fast time constant of 151 ± 12.3 ms and a slow time constant of 7.4 ± 1.2 s. The mean and median of the dye velocity distribution were 81.7 ± 28.9 μm/s \((n = 18)\) and 20.9 μm/s \((range: 2.9 to 346.6 μm/s)\) in linear vessels and 83.2 ± 16.4 μm/s \((n = 21)\) and 62.5 μm/s \((range: 5.4 to 303.2 μm/s)\) in loops, respectively. However, the normality test showed that the frequency distribution of flow velocities was clearly asymmetrical, significantly departing from normality in both linear vessels \((P < 0.01)\) and loops \((P < 0.05)\). Therefore, to statistically compare the two data distributions, the original flow velocity values were first transformed into their corresponding logarithms to base 10, as described by a translated hyperbola of the following type: \( y = \frac{a \times b}{x + b} \), where \(a\) and \(b\) are constant, \(x\) is vessel diameter, and \(y\) is geometric mean velocity of the dye (Fig. 7C, inset). When plotted as a function of the corresponding vessel diameter, \(a\) and \(b\) were first transformed into their corresponding logarithms to base 10, and then transformed back to obtain the geometrical means of the corresponding \( \log_{10} \) distributions. The mean \( \log_{10} \) velocity was 1.4 ± 0.2 μm/s in linear vessels and 1.7 ± 0.1 μm/s in loops, respectively. When transformed back, the geometric means of the original velocity distributions were significantly lower \((P < 0.01)\) in linear vessels (26.3 ± 1.4 μm/s) than in loops (51.3 ± 3.2 μm/s).

When plotted as a function of the corresponding vessel diameter, flow velocity data were quite dispersed (Fig. 4), although they tended to exponentially decrease with increasing diameter in both linear vessels (solid circles; \(v = 8.630.3^{-0.05 \times d} \), \(n = 18\), \(r^2 = 0.60, P < 0.01\)) and loops (open circles, \(v = 1.526.4^{-0.05 \times d} \), \(n = 21\), \(r^2 = 0.43, P < 0.01\)). To test whether, despite the data variability, the two populations might differ, the original flow velocity values were transformed into the corresponding \( \log_{10} \) flow velocity values and plotted against vessel diameter. The regression lines fitted through the linear vessel \((\log_{10} v = 2.3 - 0.084 \times d, n = 18, r^2 = 0.18)\) and loop \((\log_{10} v = 2.4 - 0.0115 \times d, n = 21, r^2 = 0.15)\) data were barely significant, with no differences in either slope or elevation.

The decay rate of flow velocity plotted as a function of the corresponding vessel cross-sectional area, calculated as \(S = \pi \times (d/2)^2 \times (d/2)\), was described by a translated hyperbola of the following type: \(y = \frac{a \times b}{x + b} \), in both linear vessels \((a = -65.8, b = -242.6, r^2 = 0.56, t = 5.9, n = 18, P < 0.001)\) and loops \((a = -37.8, b = -168.1, r^2 = 0.42, t = 3.9, n = 21, P < 0.001)\).

Notwithstanding the higher variability, a hyperbolic decay seemed adequate to fit the data even when the data were pooled \((a = -368.9, b = -60.9, r^2 = 0.19, t = 2.8, n = 39, P < 0.01)\).

Lymph flow, calculated as the product of lymph flow velocity multiplied by cross-sectional area, i.e., \(V_{\text{lymph}} = v \times S = v \times \pi \times (d/2)^2 \), was not significantly different \((P = 0.89)\) in linear lymphatic vessels \((10.6 ± 3.2 \text{ nl/min, } n = 18)\) and single vessels belonging to complex loops \((6.9 ± 1.1 \text{ nl/min, } n = 21)\), with an average lymph flow rate of 8.6 ± 1.6 nl/min \((n = 39)\).

Although white light epi-illumination of the diaphragmatic surface allowed identification of most of the lymphatic mesh, the presence and positioning of unidirectional valves were not easily detectable (Fig. 1, A and B). However, in a few instances, the progression of the injected fluorescent dye bolus into a vessel was impeded by a closed intraluminal valve that obstructed the pathway, causing the accumulation of FITC-dextran at the site of the obstruction and indirectly revealing the presence of the valve itself (Fig. 6). At time of injection, a faint profile of the convolute vessel was left after the fast initial distribution of the dye (Fig. 6A). As shown in the Fig. 6A, inset, at a greater magnification, vessel dilation on the right of the small inlet vessel (arrow) suggested the presence of a unidirectional valve closed by fluid backflow. Similarly, the accumulation of the dye shown in Fig. 6B (arrow) suggested the presence of another valve, which was at first closed and opened thereafter, allowing further fluid progression into the vessel (Fig. 6, C-E).

Intraluminal valves were observed not only in linear vessels but also in loops (Fig. 7), where they seemed to control both flow direction and recirculation of fluid within the loop. In the example shown in Fig. 7, after the dye passed through a first valve (detectable in Fig. 7C), the dye oscillated back and forth for ~4 min before being squeezed forward, filling the loop counter clockwise (Fig. 7F). As shown in Fig. 7, G-I, further clockwise dye progression was hindered by a closed valve (Fig. 7G) and favored in the counterclockwise direction (Fig. 7, H and I).

Distances between any two detectable intraluminal valves were similar in linear vessels (966.9 ± 128.8 μm, \(n = 25\)) and loops (840.5 ± 194.8 μm, \(n = 17\)). The geometric mean velocity of the dye in the segments with valves averaged 18.5 ± 1.05 μm/s \((n = 6)\), which was significantly slower if compared with both linear vessels and loops \((P < 0.05)\) by ANOVA with no apparent valves.

**DISCUSSION**

The lymphatic vessels running over the diaphragmatic surface display the following typical features that allow their identification as submesothelial lacunae of the initial diaphragmatic lymphatic network: 1) they form a bidimensional mesh laying on top of muscular or tendineous diaphragmatic fibers;
they can be easily observed beneath the transparent parietal pleura covering the diaphragmatic surface (Fig. 1, A and B) even without enhancement using fluorescent markers; and 3) their average thickness is compatible with the depth of pleural and peritoneal submesothelial interstitial space (8). The diaphragmatic lymphatic network in the open-chest preparation. The experimental approach required to visualize the pleural diaphragmatic dome and micropuncture its lymphatic network surely caused many alterations of the preexisting physiological steady state: 1) when the chest is opened, pleural pressure becomes atmospheric; therefore, lung inflation needs to be supported by positive end-inspiratory alveolar pressure, which, in turn, is expected to increases pulmonary vascular resistances, reducing cardiac output; 2) since pleural pressure raised to atmospheric, the pressure difference between the peritoneal and pleural sides of the diaphragm decreases, modifying the shape of the diaphragmatic dome by increasing its radius of curvature; and 3) to micropuncture the lymphatic vessels, the diaphragm must be paralyzed, a condition that abolishes active inspiration and releases diaphragmatic skeletal muscle tone even at end expiration. Thus, the outward radial stress exerted by diaphragmatic muscular fibers on the wall of submesothelial lymphatics is expected to be smaller in the open chest than in the closed chest. A reduced radial tension plus the lack of the pulling action exerted by the subatmospheric intrapleural pressure might have decreased the caliper of the lymphatic vessels, thus increasing viscous resistances to lymph flow. The reasonable expectation that diaphragmatic lymphatic function might be impaired in the open chest is supported by the fact that diaphragmatic lymphatic pressure is significantly more subatmospheric in the intact chest than in the open chest (16). On the other hand, the potentiality of diaphragmatic lymphatics to maintain at least part of their function even in the open-chest preparation is demonstrated by the cyclic development of pressure gradients able to drive fluid from the interstitial (or pleural) compartment into the lymphatic lumen as a consequence of the cardiogenic tissue displacement (18). Therefore, provided loops and linear segments maintain their distinct functional roles in the network, we believe that the results of the present study may provide useful information on the specific role they play in spontaneous breathing. Flow velocity and lymph flow in submesothelial lacunae. Intraluminal lymph flow velocity was derived from the speed of progression of the fluorescent dye injected in the vessel, provided that the dye 1) homogenously mixed with the already present intraluminal fluid and 2) did not escape or adhere to vessel walls, at least during the recording time. These requirements were met by minimizing the volume of the injected bolus (4.6 nl) and using dextrans of approxi-
mately the same molecular weight of plasma globulins at a concentration (~2 g/dl) similar to the total protein concentration in rat pleural fluid (13). Therefore, the injected fluid was expected to alter neither the protein concentration nor viscosity of the lymph (μlymph) normally flowing in the submesothelial lacunae.

As shown in Fig. 3A, injected dextrans remained in the vessel without overt leakage even 30 min after dye injection. This phenomenon cannot be attributed to the size of dextrans (larger than albumins but much smaller than other plasma proteins and cells) but rather to the existence of so-called “primary” unidirectional valves, which have been observed in the diaphragm (8), skeletal cremaster muscle (27), and the mesentery (15). A potential overestimation of the actual lymph flow velocity might have been caused by the lymphatic pressure increase induced by the injection maneuver. However, as shown in Fig. 3B, the lymphatic pressure elevation was shortly and completely nullified while the tracer progression along the vessel was monitored up to 20–25 min after the injection. Therefore, individual flow velocities measured with the present approach ought to be essentially unaffected by the transient lymphatic pressure increase.

For μlymph ~ 1.2 × 10^{-2} Poise, the average wall shear stress (τ), calculated as τ = [(8 × μlymph × v)/d], would amount to ~0.04 dyn/cm² in linear vessels and ~0.11 dyn/cm² in convolute loops. These values are one to two orders of magnitude smaller than in prenodal phasically contracting mesenteric (5) or dermal (23) lymphatics of approximately the same size. Therefore, assuming similar μlymph and d in mesenteric and diaphragmatic vessels, flow velocity would be much lower in diaphragmatic lymph vessels than in mesenteric vessels (5). This difference might reflect the proximity of the vessel to its interstitial (or pleural) origin. Indeed, the fact that lymph flow is about two order of magnitude higher in prenodal mesenteric lymphatics (5) than in diaphragmatic lacunae indicates that either total mesenteric lymph flow is much higher than diaphragmatic lymph flow and/or that prenodal mesenteric lymphatics receive and collect lymph from other smaller tributary vessels originating directly from the intestinal wall. Intraluminal flow kinetics and flow velocity might also reflect the strength of the propulsive mechanism. In soft tissues like the mesentery or dermis, lymph formation and propulsion depend on synchronized contractions of smooth muscle cells in the wall of adjacent lymphangions (1, 2, 10, 22). In thoracic tissues such a mechanisms exists, but it seems to be marginal, at least in initial lymphatics: in fact, although sequences of lymphangions separated by unidirectional valves are detectable in Figs. 2, 6, and 7 and were observed also in previous studies in rabbits (16) and rats (18), very little morphological evidence of smooth muscle cells was found in the wall of mesentery and interscalenic (8). In line with these observations, lymphatic pressure waves supported by the spontaneous contraction of lymphatic smooth muscle cells can only rarely be recorded in diaphragmatic and intercostal lymphatic networks (16). In fact, in thoracic tissues,
lymph formation and progression seem to rely mostly on compression or expansion of the lymphatic vessel lumen caused by external forces developing across the vessel wall when the tissue is displaced by the beating heart or by the contraction/relaxation of respiratory muscles (14, 16, 18). Finally, one cannot exclude that the differences in flow velocity between diaphragmatic and mesenteric or dermal lymphatics might simply reflect their specific function in the lymphatic network: in fact, whereas prenodal mesenteric lymphatics behave as collecting propulsive ducts, submesothelial lymphatic lacunae seem to serve as collecting capacitance reservoirs, receiving fluid from stomata and draining into deeper collecting ducts (8).

Total diaphragmatic lymph flow, derived in sheep by evaluating the effect of severing efferent diaphragmatic lymphatics on the caudal mediastinal node lymph flow, ranges between 0.4 and 3 ml/h (4, 6). The lower estimate, obtained at steady state 6 h after acute surgery (4), more closely reflects the physiological value and, assuming a sheep body weight of ~30 kg, corresponds to a diaphragmatic lymph flow of ~90 nl/min in a 400-g rat. For a steady-state pleural fluid turnover of 0.019 ml·kg⁻¹·h⁻¹.

Fig. 7. A–I: sequence of stereomicroscopic images taken after the injection of a bolus of FITC-dextran at the bottom left corner of a convoluted lymphatic loop. The injecting pipette is visible on the left side of A and B and was subsequently withdrawn. After a small section of the loop was filled (B), the dye oscillated back and forth without being able to enter the circular loop, which was occluded by a closed valve (C–E). At ~4 min from injection (F), the valve opened, leaving the dye flowing counterclockwise through the entire loop (H and I). Scale bar in A = 100 µm for all images.
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and since ~40% of the lymphatic pleural fluid drainage occurs through the diaphragmatic network (19), >50% (~50 nl/min) of the diaphragmatic lymph flow would derive from drainage of the pleural fluid.

Linear vessels and loops. Although belonging to the same hierarchic order of lymphatics, linear vessels and loops may be distinguished based on their distribution on the diaphragmatic surface and on intraluminal flow kinetics: 1) linear vessels run over both the muscular and tendineous regions of the diaphragm, whereas loops are preferentially observed at the diaphragmatic muscular periphery; 2) lymph flow was similar in linear vessels and individual vessels of a loop; therefore, one would expect total lymph flow through the entire loop, where multiple vessels are placed in parallel, to be higher compared with single linear vessels; and 3) the motion pattern of an injected dye was very different in the two vessels.

After being injected into linear vessels (Fig. 6, A–E), FITC-dextran progressed unidirectionally from the peripheral to the ventromedial zone of the diaphragm. Very different flow kinetics were instead encountered in convoluted loops (Fig. 7, A–I), where the FITC-dextran progression was always counterclockwise, with complex motion patterns, apparently requiring afferent duct squeezing and the presence of a luminal unidirectional valve system.

All of the above considerations suggest that loops might serve as collecting basins, receiving fluid directly from the pleural (or peritoneal) stoma to subsequently redistribute the lymph to larger linear vessels running toward the tendineous medial portion of the diaphragmatic dome and/or to transverse and collecting ducts (8) running deeper in diaphragmatic tissue. Lymph formation in loops might be enhanced during expiration, when the tone of diaphragmatic muscular fibers is low and liquid pressure in the pleural region facing the diaphragm, although subatmospheric, is higher than at end inspiration (11). During inspiration, muscular fibers contract and shorten while the medial tendineous fibers are stretched, a condition that might favor squeezing of the lymph from loops into linear vessels.

Intraluminal valves. Fluid recirculation into, within, and out of the loops as well as the pump-conduit behavior of lymphangions both in loops and linear vessels requires the existence of unidirectional valves with well-regulated opening/closing patterns. Unlike what was observed in mesenteric lymphatics, where the localization of intraluminal valves at the conjunction between adjacent lymphangions was detected at stereomicroscopic magnification through the soft and transparent mesentery (5, 29, 30), intraluminal valves were only rarely detectable under stereomicroscopic epi-illumination of submesothelial lymphatics. In fact, unlike mesenteric lymphatics, diaphragmatic lacunae I) are mostly devoid of smooth muscle cells, which may be found, although sparsely distributed, in the walls of deeper, not-visible collecting lymphatic ducts (8); 2) are tightly connected to extracellular matrix macromolecules (8) that, even if lymphangions contract, would limit the inward displacement of the lymphangion wall; 3) are covered by parietal pleura and the subpleural interstitial space, which limit, even at high stereomicroscopic magnification, the visibility of lymphatic lumen details (Fig. 1, A and B); and, finally, 4) lay over a muscular plane continuously displaced by cardiogenic swings, which blurs the image, reducing accurate analysis of its details. Intraluminal valves were occasionally indirectly revealed after an intraluminal injection of fluorescent dye to measure flow velocity, as shown in Figs. 2, 6, A–E, and 7, A–I. A detailed and quantitative description of the behavior of intraluminal valves in submesothelial lacunae is beyond the target of the present study. However, the complex pattern of flow invasion into linear vessels or loops suggests that lymphatic valves might not only set flow direction through the channel but also behave like resistive low-velocity gates, isolating the entire loop from or connect it to the inlet and outlet linear vessels and modulating recirculating fluid fluxes.

Conclusions. The pattern of fluid distribution within the diaphragmatic submesothelial lacunae suggests that lymphatic loops in the muscular periphery might be the preferential sites of fluid entrance into the diaphragmatic lymphatic network; this role might be enhanced at end expiration when muscle fibers are relaxed, pleural liquid pressure is the least negative throughout the respiratory cycle, and a favorable pleural-to-lacunae pressure gradient develops. Larger linear vessels encountered mainly in the medial diaphragmatic tendon seem instead to be more specifically involved in conveying lymph out of the diaphragm on inspiration, when contraction of the peripheral muscular fibers squeezes the lymph from the loops. Therefore, the spatial organization and geometrical arrangement of the diaphragmatic submesothelial vessels seem to be finalized at exploiting the contraction/relaxation phases of diaphragmatic muscle fibers to alternatively enhance lymph formation or progression into the lymphatic network, thus contributing to optimize fluid and solute removal from the pleural and peritoneal cavities throughout the entire respiratory cycle.

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