The Position- and Lymphatic Lumen-Controlled Tissue Chambers to Study Live Lymphatic Vessels and Surrounding Tissues *ex Vivo*

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Abstract

Background: Until now, there has been no tool available to provide lymphatic researchers the ability to perform experiments in tissue explants containing lymphatic vessels under tissue position- and lymphatic lumen-controlled conditions.

Methods and Results: In this article we provide technical details and description of the method of using the newly developed and implemented the position- and lymphatic lumen-controlled tissue chambers to study live lymphatic vessels and surrounding tissues *ex vivo*. In this study, we, for the first time, performed detailed comparative analysis of the contractile and pumping activity of rat mesenteric lymphatic vessels (MLVs) situated within tissue explants mounted in new tissue chambers and isolated, cannulated, and pressurized rat MLVs maintained in isolated vessel setups. We found no significant differences of the effects of both transmural pressure- and wall shear stress sensitivities of MLVs in tissue chambers and isolated MLVs.

Conclusions: We conclude that this new experimental tool, a position- and lymphatic lumen-controlled tissue chamber, allows precise investigation of lymphatic function of MLVs interacting with elements of the tissue microenvironment. This method provides an important new set of experimental tools to investigate lymphatic function.

Introduction

THE LAST TWO DECADES have seen a growing of interest in studying the fundamental regulatory mechanisms of lymphatic function, therefore providing a deeper theoretical basis for new advancements in treatment of lymphatic-related diseases.^{1–3} Despite general progress in development of available cardiovascular research tools and experimental techniques, studies in experimental lymphology have their own unique difficulties that must be accounted for by lymphatic researchers. Such problems are often related to difficulties in visualization of lymphatic vessels and lymph flow. As a result, the development of research tools and approaches in modern lymphatic biology to some degree has lagged behind the advances in cardiovascular biology. The difficulty in visualizing of the lymphatic vessels mainly relates to their small sizes, thin walls, and lack of red blood cells resulting in almost colorless and transparent lymphatic vessels. Additionally their frequent location deep within adipose tissue, combined with tight connections of the

fragile lymphatic wall to the elements of surrounding tissue microenvironment, further impair manipulation of the vessels. Even small amounts of mechanical damage of lymphatic vessels tissue, which are also very sensitive to introduction of tracers inside of them,^{4,5} may create serious impairments in measuring various parameters of lymphatic function and create complications in maintaining lymphatic specimens viable in *ex vivo* settings.

For experimental lymphology, this means that some experimental approaches are primarily based on use of isolated lymphatic preparations, which requires a comparatively long period of time to achieve advanced levels in dissection/handling skills. Another method commonly applied to the physiological study of lymphatic vessels is *in situ* imaging, which adds technical challenges when imaging the vessels and in some cases requires imaging modalities that are prohibitively expensive for most researchers.

Despite these difficulties, the isolated lymphatic vessel techniques has provided and will continue to provide necessary

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biological information on the regulatory mechanisms of lymphatic function.⁶ Recent studies⁷ directly confirmed that isolated lymphatic vessels experiments recapitulate normal contractile function of the same vessels observed under control conditions *in vivo*.^{8,9} At the same time, the regulatory role of the interactions between cellular elements of surrounding tissue microenvironment and lymphatic vessels cannot be neglected, especially when normal alterations of the microenvironment (i.e., healthy aging⁷) are disrupted under pathological conditions.^{10,11}

We recently demonstrated that function of aged mesenteric lymphatic vessels (MLVs) depends on the status of some cellular elements located in close proximity to those lymphatic vessels.⁷ We hypothesized that mast cells, which are concentrated in higher density by MLVs than the surrounding tissue, may exist at certain levels of basal activation in aged tissues and release several lymphatic contractions-stimulatory substances.^{12–21} These substances released from the mast cells may stimulate contractility of aged MLVs, counterbalancing therefore inhibitory effects of the excessive NO production within aged lymphatic vessels.²²

To test this hypothesis, we developed a new experimental tool, specialized tissue position-controlled chambers, which allowed us to maintain viable live explants of mesenteric tissues containing lymphatic vessels. These chambers permitted us to perform several types of experimental manipulations on these tissues, including multiple microscopic imaging of the same segments of mesentery while maintaining the exact location of selected zones in the microscope's field of view. Using these chambers in combination with epifluorescent and bright field microscopy of the same specimens, we confirmed the presence of mast cells by MLVs in higher density.²³ The chambers also allowed us to compare the levels of basal activation of these mast cells in live adult and aged mesenteric tissues, proving that there are higher levels of basal activation of aged mast cells.²³ These findings were key to explaining our previous findings.⁷

Moreover these tissue chambers allowed us to perform treatments of mesenteric explants by various biologically active substances and subsequent multiple imaging to confirm that pre-existing aging-related basal activation of mast cells located by MLVs results in reduced ability of aged mast cells to respond to acute stimulation.²³ Consequently, we also found that mast cell-dependent recruitment of MHC class II positive cells and eosinophils towards MLVs is diminished in aged mesentery.²⁴ Therefore our previous publications^{23,24} clearly demonstrated that tissue explants are viable inside the new tissue position-controlled chambers.

However, in our studies mentioned above, the MLVs within mesenteric explants remained clamped (i.e., closed input and output ends) and lacked controlled intraluminal pressure. Although in many live specimens we visually observed lymphatic contractility, the viability of MLVs in our chambers have not been confirmed, and the contractile and pump functions of these lymphatic vessels located in tissues explants have not yet been evaluated. The purpose of this study was to determine if our newly developed experimental tool will allow lymphatic vessels under live conditions to be cannulated to control pressure and flow within these vessels, and to measure their function while remaining viable.

Materials and Methods

Animals and surgery

We used Sprague-Dawley male rats (weighing between 200 and 300 g). All animal procedures were reviewed and approved by our Institutional Animal Care and Use Committee and were in accordance with federal and local regulations.

To isolate the segment of mesentery including the MLVs, the rats were anesthetized with a solution containing a combination of fentanyl/droperidol (InnovarVet® 0.3 ml/kg IM) and diazepam (2.5 mg/kg IM). A 4-cm long midline abdominal incision was made and the whole gut was exteriorized and removed by cutting the root of the mesentery after clamping the superior mesenteric artery and vein in order to prevent excess bleeding. After this procedure, the rats were euthanized with nembutal sodium solution (120 mg/kg body weight IC) and verified by thoracotomy. The exteriorized gut with mesentery was rinsed several times in PBS (catalog #6505; EMD Chemicals, Gibbstown, NJ) and pinned down on a sylgard-coated 150-mm tissue culture dish. Under the stereomicroscope, we identified a section of mesentery with MLV that was not covered with adipose tissue, and using scissors we removed approximately a 4×4 cm segment of mesentery containing the MLV.

Setting mesenteric explants into position-controlled tissue chambers

The goal of this experimental preparation was to arrange tissue chambers with cannulated and pressurized MLVs within mesenteric explant on the stage of microscope, as demonstrated on panel A of Figure 1. This panel demonstrates the position- and lymphatic lumen-controlled mesenteric tissue chamber in its full assembly and final position with glass micropipettes secured in pipette holders; one shown as an example (1) of micromanipulators (2) and inserted into the lumen of a MLV within mesenteric explant mounted inside tissue chamber (3). The tissue chamber fit into a mount in the middle of the custom machined microscope stage top (4). Panel B of Figure 1 demonstrates all parts of tissue chamber in the disassembled state. The ring-shaped stainless steel base (5) fits into the rounded opening of the microscope stage and contains three fixed threaded studs. The bottom part of the tissue chamber (6) inserts inside the steel base (5) which has a rounded cover slip glued into a recess in the bottom and two side holes that allow inserting luer adaptors to connect tubing to superfuse space under mesenteric tissue explant. Tissue explant must be accurately positioned on part (6) filled by saline and then clamped in position by the top part of the tissue chamber (7) and tightened by thumb screws to the base (5) and the assembled chamber is shown on panel C of Figure 1. Any tissue can be removed by iris scissors after mounting. The inner space of the part (7) is large enough to allow superfusion of the space above the mesenteric tissue explant and to insert pipettes into MLV. View of an assembled chamber from the bottom is shown on panel D of Figure 1.

The purpose of this report is to explain the principles of usage and to demonstrate viability of using MLVs inside of tissue explants for measurements of the contractile and pump functions; we compared the activity of the vessels in chambers to isolated MLVs. As the dimensions and needs of

FIG. 1. Parts and an example of usage of the position- and lymphatic lumen-controlled tissue chambers to study live lymphatic vessels and surrounding tissues *ex vivo*. (A) Tissue chamber under microscope. 1. pipette holder with micropipette inserted into lymphatic vessel; 2. micromanipulator; 3. tissue chamber set in the middle of the custom made microscope stage top (4). (B) Parts of tissue chamber. 5. stainless steel base; 6. bottom part of tissue chamber; 7. top part of the tissue chamber. (C) and (D) Tissue chamber in its full assembly (side view and bottom view corresponding). (E) Screen capture of the diameter tracking computer program with an example of MLV inside mesenteric tissue and diameter tracking window in analog mode.

laboratories vary, we are not providing the dimensions of the chambers. These dimensions should be modified by the end user depending on the type of microscope used. Those who wish to replicate this chamber exactly may contact the corresponding author for dimensions.

Cannulation of MLVs in tissue chambers

Each excised mesenteric segment containing MLV was rinsed in PBS in a separate dish and then transferred to the tissue chamber to its fixed position with MLV positioned in the center of the chamber. The bottom and top compartments of the tissue chamber were filled with pH stabilized DMEM/F-12

(catalog #11039-021; Life Technologies, Grand Island, NY, USA). pH stabilization of DMEM/F-12 was achieved by incubating opened bottles for approximately 12 h at 38°C and 10% CO₂ incubator. Two resistance-matched²⁵ glass micropipettes (the diameter of the tip was 40–70 μm) were attached to the micromanipulators (model YOU-3, Narishige, Tokyo, Japan), connected to tubing, and filled by pre-warmed DMEM/F-12. YOU-3 micromanipulators have magnets incorporated to their bases allowing them to be stably fixed on the stainless steel microscope stage and to align their position with location of visible part of MLV inside tissue explant. At this point, the tissue chamber with segment of mesentery and the microscope stage top was transferred to a stereomicroscope.

FIG. 2. Comparative analysis of contractile parameters of the MLVs located in tissue chambers and isolated MLVs at various levels of transmural pressure. (A) normalized outer diameter; (B) normalized contraction amplitude; (C) contraction frequency; (D) fractional pump flow. * indicates significant differences ($p < 0.05$) between lymphatic contractile parameters within each vessel group at transmural pressure 1 cm H₂O and at other transmural pressures.

Next, we punctured the glass micropipettes into the upstream and downstream ends of the visible MLV within tissue explant under a stereomicroscope. Therefore one micropipette served as input pipette (according to natural direction of lymph flow determined by position of lymphatic valves), and the other as output pipette. Care was taken to ensure that there were no air bubbles in the tubing or the pipettes. Confirmation of the micropipettes seal in the wall of MLVs was done by raising the tubing connected to the input end of the micropipette and observing increased vessel diameter and no vessel slippage.

After cannulation of the MLV under the stereomicroscope, the whole assembly (tissue chamber with mesenteric explant, micropipettes positioned in manipulators, and custom-made microscope stage) was transferred to the stage of an inverted microscope Olympus CKX41 with great care not to displace the pipette from the MLV. The inflow and outflow pipettes were connected to independently adjustable pressure reservoirs filled with pH stabilized DMEM/F-12. The MLVs were set to an equilibration transmural pressure of 5 cm H₂O at 38°C for 60 min. Once tone and spontaneous contractions were observed, the vessels were equilibrated by setting a transmural pressure of 3 cm H₂O for another 30 min prior to beginning the experiment. During all experiments, mesenteric tissue segments were constantly superfused from top

and bottom with pre-warmed pH stabilized DMEM/F-12, and the tissue chamber was also constantly warmed to maintain the temperature of the pH stabilized DMEM/F-12 at $38 \pm 0.1^\circ\text{C}$. A CCD video camera (model KP-D20B4, Hitachi Ltd., Tokyo, Japan), hi-resolution monitor (TM-A13SU, JVC, Wayne, NJ, USA) and DVD/HDD recorder (DVR-560H, Pioneer, Tokyo, Japan) was connected to a Windows-operated computer equipped with a National Instruments data acquisition card (NI PCI-1410). This arrangement was used to observe, video record, and to track the diameter of MLVs continuously during all experiments.

To compare parameters of contractility of MLVs mounted within tissue chambers with contractility of isolated MLVs (experimental technique which recapitulates contractility of MLVs *in vivo*⁷), we also performed experiments with isolated MLVs obtained from the similar animals. We used a well-described experimental technique for measuring function in isolated lymphatic vessels.^{4,11,25–27} One deviation from the previously described protocol was the use of DMEM/F-12 in place of albumin containing physiological salt solution.

Diameters of MLVs situated in mesenteric tissue explants were tracked continuously during experiments using “Vessel Track” software developed previously.²⁸ Figure 1, panel E, demonstrates the screen capture of the diameter tracking computer program with an example of a MLV inside mesenteric

FIG. 3. Comparative analysis of contractile parameters of the MLVs located in tissue chambers and isolated MLVs during the increases in imposed flow. (A) normalized outer diameter; (B) normalized contraction amplitude; (C) normalized contraction frequency; (D) normalized fractional pump flow. * indicates significant differences ($p < 0.05$) between lymphatic contractile parameters within each vessel group at no imposed flow conditions, and conditions with presence of various imposed flow gradients.

tissue and the diameter tracking window in analog mode. We used cardiac pump analogies to define systole and diastole in reference to the lymphatic contractile cycle,^{8,27,29} and the end-diastolic and end-systolic points in the diameter tracings were recorded for each 5-min interval for the transmural pressure of 1, 3, and 5 cm H₂O and for imposed flow gradients of 0, 1, and 5 cm H₂O (at transmural pressure of 5 cm H₂O). This allowed us to determine transmural pressure- and wall shear stress-sensitivity of MLVS within tissue explants.

At the same time we performed experiments with isolated MLVs obtained from similar animals. For these experiments, we used previously described techniques to measure contractile and pumping activity of isolated lymphatic vessels.^{25–27,30} We performed isolated MLV experiments using DMEM/F-12 and recorded their activity under the same conditions as for MLVs situated within tissue explants. This allowed us to directly compare function of MLVs in tissue chambers and isolated MLVs.

From the records of the lymphatic end-diastolic and end-systolic diameters (EDD and ESD), the following lymph pump parameters were calculated: normalized outer diameter (EDD, expressed as a percentage of the EDD at transmural pressure of 1 cm H₂O (for transmural pressure-sensitivity set of data) and at imposed flow gradient of 0 cm H₂O (for wall shear stress-sensitivity set of data), contraction amplitude

(the difference between the EDD and ESD, expressed as a percentage of the corresponding EDD), contraction frequency (/minute) (expressed as a percentage of the contraction frequency at imposed flow gradient of 0 cm H₂O (for wall shear stress-sensitivity set of data), ejection fraction (EF, the fraction of end-diastolic volume ejected during the single lymphatic contraction, calculated using the formula $EF = (EDD^2 - ESD^2) / EDD^2$),^{2,8} and fractional pump flow (FPF, an index of lymph pump flow, calculated as the ejection fraction times the contraction frequency) [expressed as a percentage of the FPF at imposed flow gradient of 0 cm H₂O (for wall shear stress-sensitivity set of data)]. Normalizations as described above allowed us to compare responses of isolated MLVs and those situated in tissue explants at the corresponding transmural pressures and imposed flows avoiding the influence of the anatomical variations in sizes between lymphatic vessels.

Statistical differences were determined by ANOVA, regression analysis, and Students *t*-test (JMP software version 5.0.1.2. for Windows, SAS Institute Inc., Cary, NC, USA) and considered significant at $p \leq 0.05$. Only one vessel segment was used from one animal for tissue chambers group, and one vessel per animal for the isolated MLVs group. We used data from 6 animals for the functional analysis for each group.

Results

Figure 2 demonstrates the results of analysis of contractile parameters of the MLVs located in tissue chambers and isolated MLVs during changes in transmural pressure. We did not find any statistical differences in any of the four parameters of contractile activity of MLVs at any pressure point. MLVs in both conditions demonstrated similar changes in diameter, contraction amplitude, and frequency and pumping (FPF) during the increases in transmural pressure. The stretch-induced reactions of mesenteric lymphatic vessels are well characterized and MLVs in our study matched the previously described paradigms.^{26,30–34}

Figure 3 demonstrates the results of analysis of contractile parameters of the MLVs located in tissue chambers and isolated MLVs during increases in imposed flow. Again we did not find any statistical differences in any of the four parameters of contractile activity the MLVs. MLVs in both conditions demonstrated a similar degree of relaxation and inhibition of contraction amplitude and frequency and pumping (FPF) during the increases in imposed flow within them. This matches expected results for wall shear stress-induced vessel relaxation and inhibition of pumping activity in MLVs.^{11,25,26,30,33,34}

Discussion

This article provides description and experimental validation of the effectiveness of a new experimental tool, - the position- and lymphatic lumen-controlled tissue chamber, to study live lymphatic vessels and surrounding tissues *ex vivo*. Vessels stretch and wall shear stress are the two major physiological regulatory mechanisms of lymphatic transport function, which is necessary for the lymphatic system to transport immune cells, lipids, and fluids. We have presented data on experimental comparisons of function of the MLVs situated inside the mesenteric explants within these chambers and isolated, cannulated, and pressurized MLVs. We found that there were no significant differences between the two experimental conditions for both stretch- and wall shear stress-sensitivity of MLVs. Therefore measurements of lymphatic function using the new tissue chambers can recapitulate the function of isolated lymphatic vessels under control conditions and therefore lymphatic vessels *in situ*.⁷ We had already shown the effectiveness of this new experimental tool to investigate the function and immunological status of tissues located in close proximity to lymphatic vessels.^{23,24}

The benefits of the using this type of chamber relate to the comparative inexpensiveness and simplicity of design, as well as reduced vessel manipulation. The relative inexpensive manufacture allows a laboratory to utilize many chambers for large-scale experimentation. It is also possible to use them for long-term duration experiments requiring incubation of the tissue explants. The chambers are also easy to clean and repair. In addition, the volumes of the upper and lower compartments for superfusion of tissue explants are relatively low (compared to *ex vivo* isolated vessel chambers), which supports cost-effectiveness of the chambers when using expensive materials (e.g., to perform experiments with tissue transfections or with induced lymphangiogenesis). In conclusion, this new experimental tool allows precise investigation of lymphatic function of MLVs interacting with elements of tissue microenvironment

located in close proximity to them, and increases the set of experimental tools available to investigate lymphatic function.

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Author Disclosure Statement

For all authors, no competing financial interests exist.

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