Lymphology 36 (2003) 111-119

FUNCTIONAL IMPACT OF LYMPHANGIOGENESIS ON FLUID TRANSPORT AFTER LYMPH NODE EXCISION

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ABSTRACT

When a lymph node is excised, lymphangiogenesis occurs to maintain flow in the affected area. However, a complex network of small vessels replaces the node and these newly formed vessels might increase resistance to lymph transport. To test this in sheep, the popliteal lymph node from one hind limb was removed surgically. The contralateral node was left intact. After 4 to 6 weeks (a period that allowed regenerated vessels to restore flow), a prenodal lymphatic vessel in each limb was cannulated with a polyethylene catheter to permit saline infusion into the node or lymphatic regeneration site. Infusion pressures were monitored from t-pieces inserted between the infusion pump and the point of entry of the catheters in the prenodal ducts. We observed that the flow rate versus perfusion pressure relationships were significantly different in the 2 experimental preparations (node intact limbs, n=13; node excised limbs, n=10). In the limbs undergoing lymphangiogenesis, much higher infusion pressures were required to generate a given flow rate. Additionally, the regenerated lymphatic network provided a significantly increased resistance to flow. The data suggested that lymphangiogenesis restored fluid continuity to some extent in the area occupied originally by the popliteal lymph node. However, the transport properties exhibited by the newly formed lymphatics were insufficient to restore flow parameters to their original state.

Several recent developments have important implications for lymphedema research. These include the discovery of molecular regulators of lymphangiogenesis, the detection of markers that can be used to distinguish lymphatic vessels from their blood vascular counterparts and the identification of several genes that play a role in primary lymphedemas (1-4). However, key physiological issues remain to be elucidated concerning the application of molecular therapies to the lymphedema patient. Among these are questions related to the functional properties of newly formed lymphatic vessels. Whether appropriate tissue drainage can be restored following pharmacologically induced lymphangiogenesis has yet to be established convincingly.

When a lymph node is excised, lymphangiogenesis occurs and in many cases, the interstitial drainage capacity of the newly formed lymphatic vessels appears to be sufficiently developed to prevent the clinical manifestations of edema. However, are lymph transport parameters normal following lymphangiogenesis? The answer to this question may be important since it is conceivable that a relatively subtle sub-clinical change in lymph transport could alter tissue drainage such that the risk of developing lymphedema is increased.

The physiological parameters associated with lymphangiogenesis are best studied in a species large enough to permit easy access to lymph nodes and lymphatic vessels. In this

regard, sheep have been used extensively for studies on the lymphatic circulation. Indeed, the lymphangiogenesis following lymph node removal in this species has provided an important research model for the collection of lymph that has not passed through a lymph mode. Cellular immunologists remove the node of interest and after a suitable period of time, the lymphatic vessels regenerate and re-establish fluid continuity. At this point, catheters can be inserted into the larger duct that was originally downstream of the node rather than in one of the smaller prenodal vessels (5,6). The advantage of this preparation is that one can investigate the transit of lymphocytes through the tissues directly without the contaminating influence of the lymph node.

This procedure can be adapted for studies on lymphangiogenesis. Afferent lymphatic vessels leading to the popliteal lymph node arc identified easily and are of sufficient size to allow cannulation of the vessels. One can remove the node and after a suitable period of time, investigate the transport parameters associated with the regenerated vessels. In this report, we used this approach to make quantitative comparisons in fluid transport between lymphatics that form in response to the removal of the popliteal lymph node and the normal vessel network that exists when the node is intact.

MATERIALS AND METHODS

All experiments outlined in this proposal have been approved by the ethics committee at Sunnybrook and Women's College Health Sciences Centre and conform to the guidelines set by the Canadian Council on Animal Care and the Animals for Research Act of Ontario.

Popliteal Lymph Nodectomy

Sheep were anesthetized initially by IV injection of sodium pentothal. Subsequently 1.5-2.5% halothane was delivered through an endotracheal tube via a Narkomed 2 respirator for surgical maintenance. The flank of one leg was shaved and prepped with alcohol and betadine. Under sterile surgical procedures, an incision was made through the skin and the muscle separated, exposing the popliteal node. The node was dissected carefully and cleared of excess tissue and the artery and vein tied with a silk ligature before extraction. The skin was sutured with 2-0 silk using interrupted stitches and a topical antiseptic (Boroform spray) was applied to the exterior incision. Temgesic was given immediately post surgically. and as required thereafter to treat post-surgical pain. In most cases, the contra-lateral node was left intact. In a few sheep, both popliteal nodes were removed or left intact. Antibiotic (Duplocillin) was administered I.M. 3 days prior to, the day of and 3 days after surgery.

Visualization of Regenerated Lymphatic Vessels Following Removal of the Lymph

While it would be possible to utilize some of the molecular markers to identify lymphatics in sheep, these are generally unproven in this species. However, there are simpler methods to identify lymphatics using Evans blue dye and confocal microscopy. Evans blue dye was injected subcutaneously just above the hoof of the animal and after 30 minutes the tissue was harvested. The dye binds to protein and is taken up by the prenodal lymphatics, which can be observed using fluorescence microscopy.

Following tissue perfusion of the dye, a block of tissue encompassing the node-regeneration site was excised and fixed in 10% formalin at 4°C overnight. The tissue was cryoprotected by soaking in 30% sucrose solution overnight at 4°C. After this, the tissue was embedded in Tissue-Tek O.C.T. compound to bind the tissue to the specimen block and surround and cover the tissue sample. The tissue was frozen using dry ice and cut into 200 µm thick sections using a

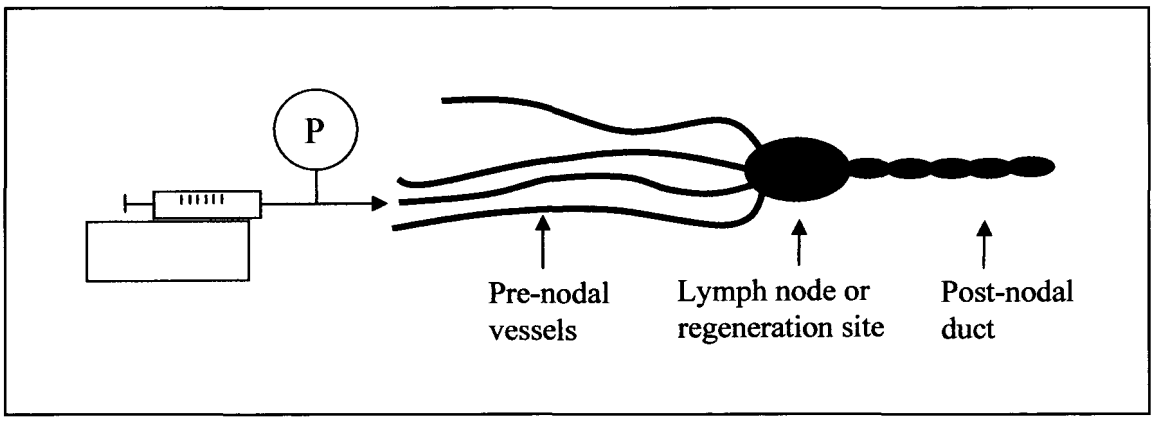

Fig. 1. Schematic illustrating the experimental design and the anatomical orientation of the perfusion protocol. P = pressure transducer.

sliding microtome. Slices were floated in PBS to uncurl and mounted onto slides. Mounting media (PVA DABCO) was used to coverslip the samples. All slides were stored at 4°C until the coverslipping media is dry.

When outlining the lymphatic vessels with Evans blue dye, a single-track configuration was employed using a Zeiss Axiovert 100M laser scanning confocal microscope. Evans blue dye was maximally excited at 550 nm, with peak emission at 610 nm. The helium/neon laser was used to excite the dye with a wavelength of 543 nm. A variety of mirrors and filters were used to detect all emission of the dye above 585 nm, thus resulting in the Evans blue red fluorescent tissue image.

Infusion Studies

Four to six weeks after lymph node removal, the animals were anesthetized and an incision made in the skin just above the hock. A small amount of Evans blue dye (0.1%) was injected into multiple sites to permit visualization of the popliteal lymphatic network. In sheep, there are usually 3-6 prenodal ducts and these enter the popliteal lymph node individually at various locations along the convex portion of the lode. One of these was exposed and cannulated in the direction of flow using a polyethylene catheter (0.96 mn OD; 0.58 mm ID). The free end of the catheter was connected to a t-piece, which in turn was attached to a syringe pump (Kd Scientific, model #260) with a short length of the same plastic tubing. The free arm of the t-piece was coupled to a pressure transducer (Cobe CDX disposable).

To avoid the potential problem that diluted blood in several forms could affect the tone of lymphatic vessels, and, consequently, might affect downstream ducts in an unpredictable manner, we used 0.9% saline as the infusate since this would have no pharmacological effects. Saline was infused into the popliteal prenodal vessel at various flow rates. At the same time, infusion pressures were recorded on a strip chart recorder (Beckman, R511A).

Experimental Protocol and Data Analysis

A schematic illustrating the experimental design is provided in *Fig. 1.*

Infusions commenced at 0.2 ml/hr and were raised in 0.2 ml/hr increments up to a maximum of 10 ml/hr. Infusate pressures were recorded continuously. The infusion rate was changed only after a steady-state

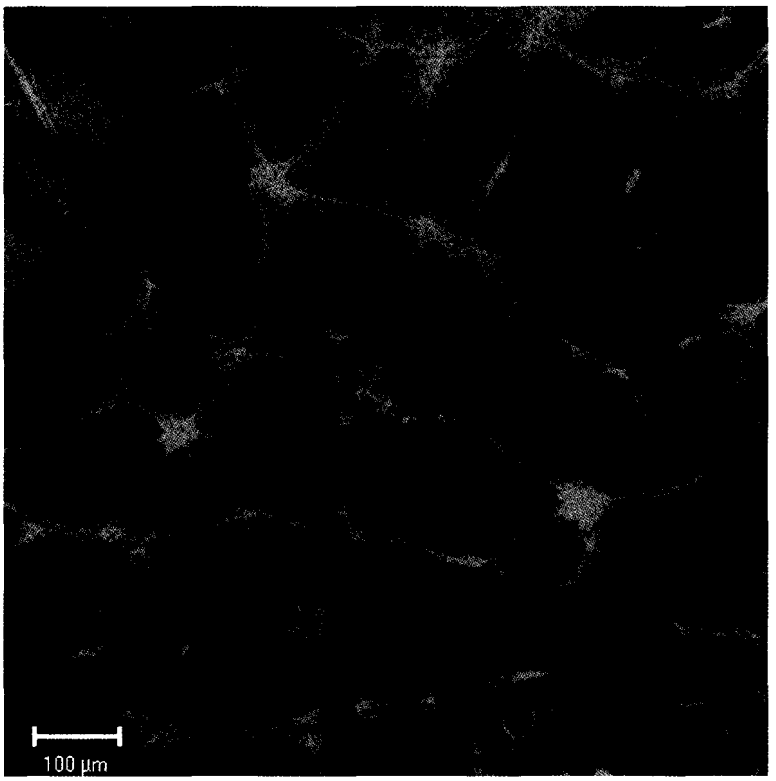

Fig. 2. Confocal image of the regenerated lymphatic network that forms in response to the removal of the popliteal lymph node. Evans blue dye (fluorescing red) was injected into the subcutaneous tissues in the drainage basin of the original node and allowed to perfuse the lymphatic vessels for 30 minutes. A plexus of fine lymphatic vessels has bridged the gap between the original afferent and efferent ducts.

pressure had been achieved (5 to 10 minutes). At the end of the experiment, the lymph node regeneration site was exposed surgically and Evans blue dye was infused through the system. In this way, we could confirm that fluid continuity had been reestablished by regenerating lymphatic vessels following lymph node removal. The average infusion pressure was plotted against the infusion rate for each experiment (node intact limbs, n=13; node excised limbs, n=10). The data was assessed with ANOVA. We interpreted $P < 0.05$ as significant.

RESULTS

Regeneration of Lymphatic Vessels after Popliteal Lymph-Excision

When a popliteal lymph node is removed, it is replaced with a plexus of small vessels that bridge the gap between the prenodal ducts and the post-nodal vessel. An example of the regenerated lymphatic network is illustrated in *Fig. 2*. That successful lymphangiogenesis between the cut ends of the vessels had been achieved at 4 weeks was indicated by the presence of Evans blue dye (injected into the drainage basin of the original popliteal node) in the lymphatic duct efferent to the regenerated site and the absence of the dye in the popliteal fossa surrounding the newly formed vessels. Additionally, at 4 weeks, there was no obvious evidence that the hind limbs of the animals were edematous suggesting that tissue drainage had been restored.

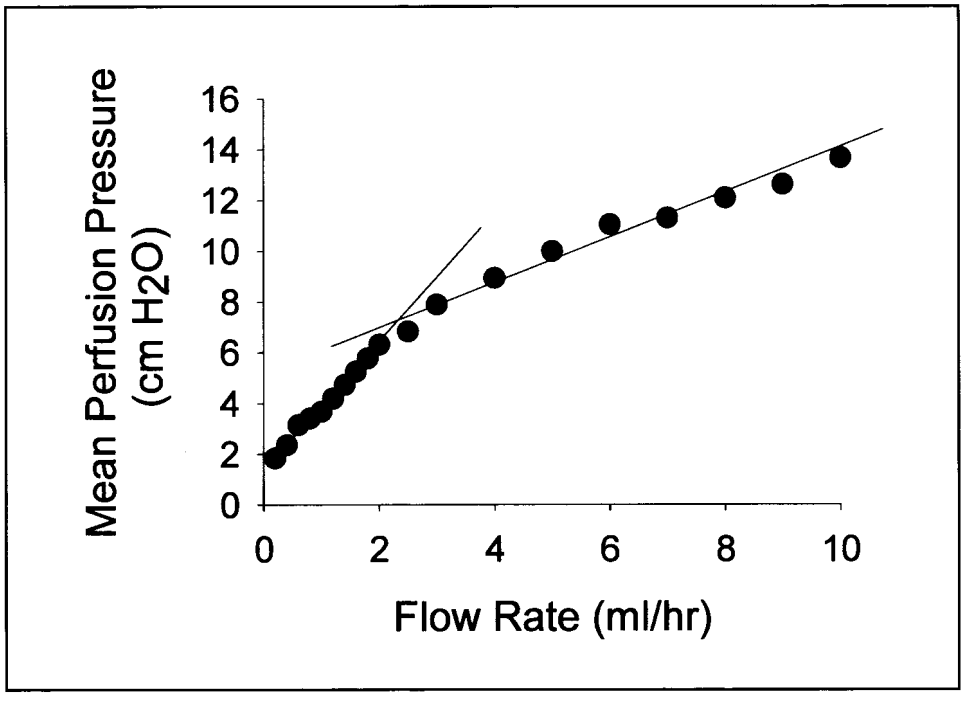

Fig. 3. Relationship between flow rate and infusion pressure. In initial experiments, the relationship was non-linear with the break-point around 2 ml/hr. This example was taken from a node-intact limb.

Pressure-Flow Relationships in Lymph Nodectomy/Regeneration Area

Initial experiments indicated a non-linear relationship between flow and perfusion pressure. An example is illustrated in *Fig. 3*. Generally, the breakpoint occurred at flow rates around 2.0 ml/hr both in node-intact and in node-excised limbs. It is possible that flow rates above 2 ml/hr open up collateral lymphatic networks perhaps leading to the pre-femoral lymph node. In any event, since normal pre-femoral popliteal lymph flow rates are generally much less than the breakpoint value, we limited subsequent infusions to a maximum 2.0 ml/hr.

Does Lymph Nodectomy/Lymphangiogenesis Alter Pressure-Flow Relationships?

Fig. 4 illustrates the averaged results from these experiments. The pressure-flow relationship observed in the node-intact limbs is illustrated by the solid circles. In the nodectomized limbs (open circles) the relationship was shifted significantly upward and appeared non-linear with a steeper initial slope up to flow of 0.4-0.6 ml/hr followed by a shallower curve beyond. *Fig. 5* illustrates the calculated resistances for each flow rate (pressure divided by flow). The lymphatic system that included the network of regenerated vessels exhibited a significantly higher perfusion resistance than that of the node-intact limbs especially at the lower rates of infusion.

DISCUSSION

As a first step in understanding the impact of lymphangiogenesis on fluid transport, we designed a perfusion study to investigate the possibility that the newly regenerated lymphatics provided a greater

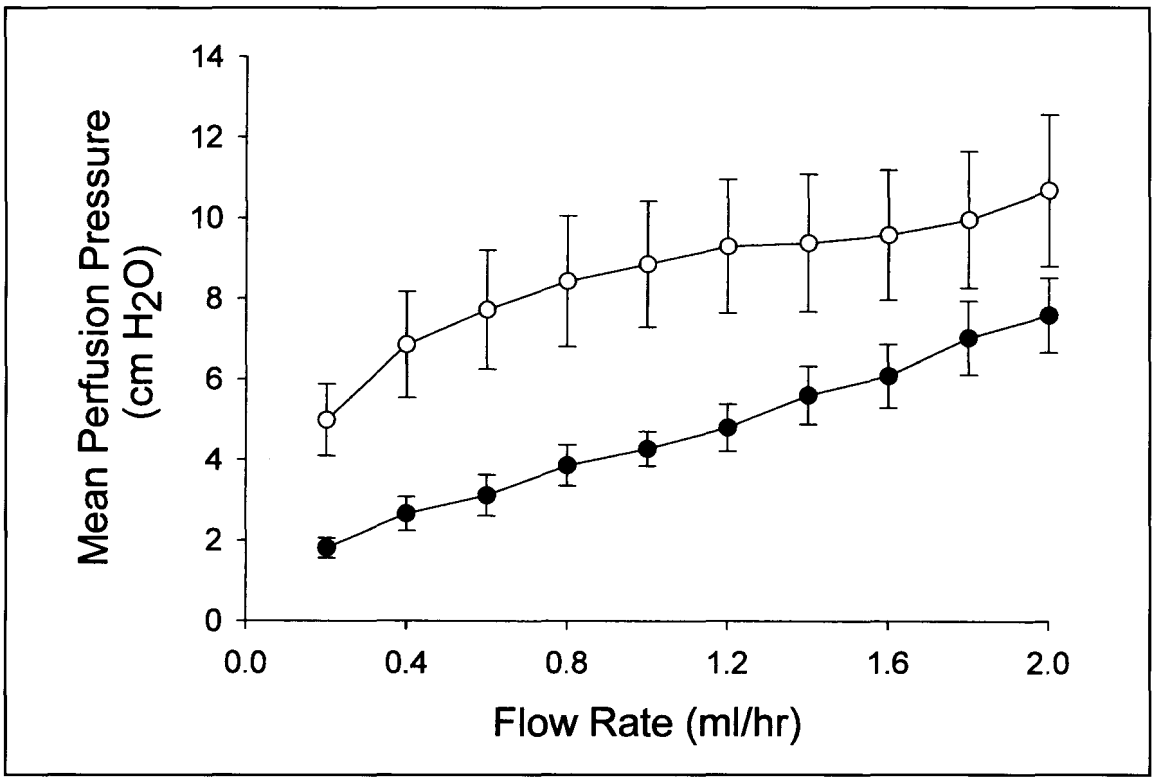

Fig. 4. Relationship between flow rate and infusion pressure. Closed circles represent averaged data from node-intact limbs (n=13). Open circles illustrate averaged data from node-excised limbs (n=10). Analysis with a 2-way ANOVA (within factor, flow; between factor, group) revealed a significant group (p=0.14) and flow effect (p<0.001).

resistance to flow compared with the fluid transport associated with an intact node. Before discussing the possible significance of these results, it is appropriate to acknowledge several limitations inherent in our experimental approach.

First we recognize that perfusion pressure is related to but not equal to the pressure within the lymphatic network, the latter representing the theoretical ideal. Preferably, we would like to measure pressure within the lymphatic vessel in the perfusion experiments. This was achieved in a study that investigated lymph node resistance in the dog (7). However, in dogs, afferent vessels converged into a manifold-like structure proximal to the node and this structure could be accessed with a catheter. This anatomical arrangement does not exist in the sheep popliteal system. In our preparation we decided not to interfere surgically with the prenodal collector in question at a downstream location as this could potentially alter the resistance parameters or introduce a source of leakage in the system.

Second, the lymph node is compartmentalized; dye studies indicate that cannulation of one of the prenodal lymphatics would result in only a portion of the node being perfused. We presume this is also true of the regenerated lymphatic network. However, there seems to be no practical way of getting around this problem as it would be very difficult to perfuse all prenodal vessels. Nonetheless, it is not unreasonable to assume that the portion of the node or regenerated lymphatic complex being perfused is representative of the other non-perfused area.

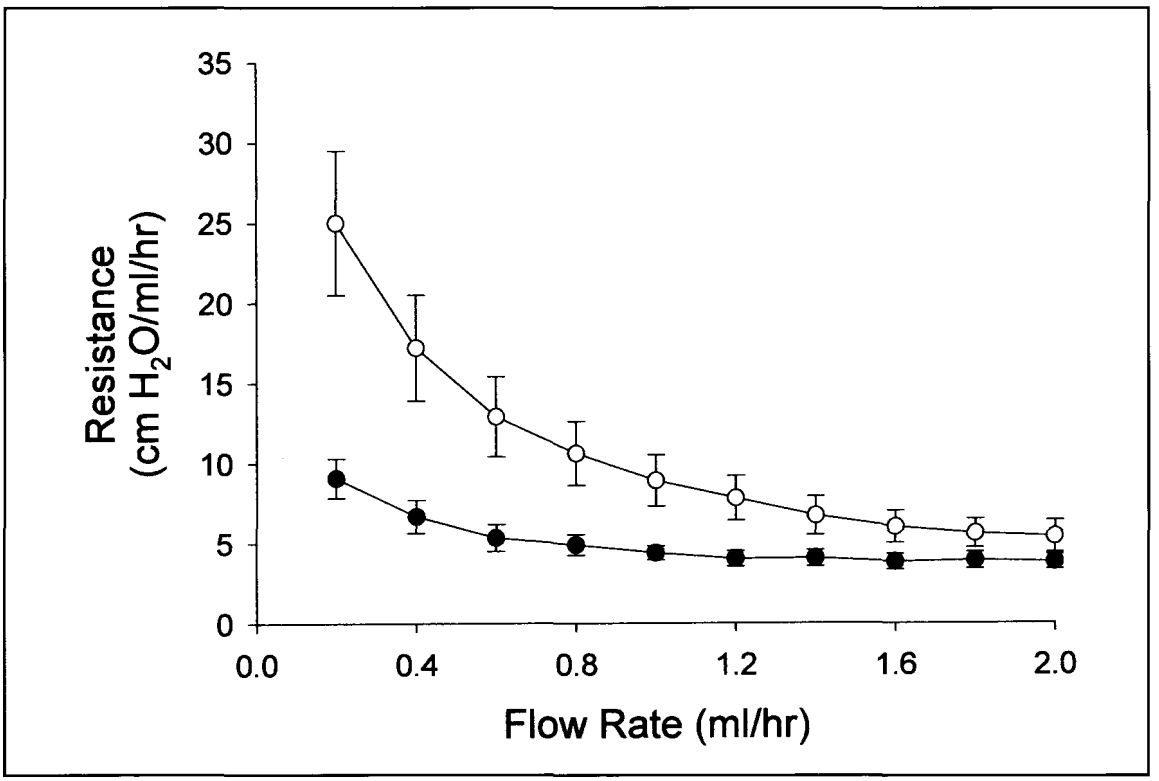

Fig. 5. Relationship between flow rate and resistance. Closed circles represent averaged data from node-intact limbs (n=13). Open circles illustrate averaged data from node-excised limbs (n=10). Analysis with a 2-way ANOVA (within factor, flow; between factor, group) revealed a significant group (p=0.005) and flow effect (p<0.001). Also noted was a significant interaction effect (p<0.001) indicating that the differences between groups were changing significantly over the range of flows tested.

Finally, we assessed the resistance to perfusion at one time only. It is possible that the flow parameters associated with lymphangiogenesis will change as the new lymphatic network matures and remodels over time. Our group will address this issue experimentally in future studies. In any event, with these limitations and assumptions in mind, the data support the following concepts.

Fluid Transport Through Newly Formed Lymphatic Vessels Is Impaired

The removal of a lymph node resulted in impaired fluid transport even though the generation of new lymphatic vessels established fluid continuity in the affected area. For a given lymph driving pressure, the flows in the limbs experiencing lymphangiogenesis were less. Additionally, in the experimental limb, a higher lymph driving pressure was needed to maintain a given flow rate. For example, in the control limb, a flow rate of 0.8 ml/hr was maintained with a lymph driving pressure a little under 4 cm H_2O. To maintain the same flow in the regenerated side a pressure around 8 cm H_2O was required.

The Newly Formed Lymphatic Network Appears to Provide a Greater Resistance to Fluid Transport

After a lymph node is removed, numerous small ducts form a vessel network

to bridge the gap that approximates a parallel, interconnecting design. Presumably this vessel architecture is advantageous since the total resistance in parallel networks is less than that of the individual vessels. However, lymphangiogenesis typically seems to produce a very irregular structure such that it is difficult to predict whether the newly formed vessel system will have an impact on lymph flow resistance. Lymph nodes are known to provide resistance to lymph transport, and this has been estimated to be 50 to 200 times greater than that provided by the lymph trunks (8,9). In our studies, we estimated the resistance of the network of newly formed lymphatic vessels and observed values that were greater than those of the original lymph node. For example, at the lowest flow rate tested, resistance of the lymphangiogenesis side was ~2 1/2 fold greater.

As has been described for dog lymph nodes (7), the resistance of the popliteal node was greatest at low flow rates and declined as flows were elevated. This is likely due to the expansion of the lymphatic channels within the node at higher pressures allowing easier passage of perfusate. A similar pattern was noted in the regenerated side, and this may also be due to dilation of the newly formed vessels. However, even though resistances declined at higher flow levels in both node-intact and node-excised preparations, the calculated values were always higher on the side in which the lymphatics had regenerated.

The development of numerous small vessels to bridge an injury-induced gap appears to be a characteristic feature of lymphangiogenesis (10), and it would appear that these irregular parallel networks provide a greater collective resistance to flow than the original lymphatic channels in the lymph nodes. We also noted that the newly formed vessels were often encased in fibrous tissue, and this may distort the lymphatic network and contribute to the elevated flow resistance.

Another factor to consider is that the fluid transport studies reported here were performed on lymphatic networks that were formed over a relatively short period. We cannot say whether the conductance or resistance parameters would improve or decline with time but this is an important issue. Clinically, lymphedema following node dissection can take many months or even years to develop. The impact of vessel remodeling and fibrosis on transport parameters needs to be investigated further.

Potential Significance

Guyton and colleagues have described the concept of a 'safety factor' that works to prevent increases in interstitial fluid volume (11). The normal negative limb interstitial fluid pressure provides some hydrostatic buffering effect, as interstitial pressure must rise before edema develops. As interstitial fluid pressure increases, lymph flow rates rise to remove fluid from the interstitial compartment. This increase in lymph flow can be up to 20 times the normal rates depending on the inciting stimulus (12). Elevated lymph flows also facilitate protein washout from the tissues decreasing the colloid osmotic pressure of the interstitial fluid (oncotic buffering effect). If lymph transport is impaired subclinically, the magnitude of this 'edema safety factor' is reduced. Under these conditions, the probability that edema formation may occur is increased because the threshold interstitial fluid pressure reflective of clinical edema would be more easily achieved if interstitial fluid pressure were allowed to rise chronically.

We do not know if the observed magnitude of resistance changes observed in this study can impact upstream tissue drainage significantly. It is of interest to note that the extrapolated y-intercept values for the regenerated lymphatic system (open circles in *Fig. 4*) were higher than those of the node intact limb. Since these intercept values represent the theoretical pressures that initiate flow, it seems likely that they are also reflec-tive of (but not equivalent to) the interstitial fluid pressure in the respective limbs.

Interstitial fluid pressure has been studied extensively in subcutaneous tissues in many species. In most cases, interstitial fluid pressure measurements in skin (including those in humans) are considerably lower than the y-intercept values noted in this study and range between 0 and -2 mm Hg (reviewed in reference 9). Pressures above atmospheric in this tissue usually reflect an edematous state. In patients with post-mastectomy edema, subcutaneous interstitial fluid pressure in affected limbs was 1.9 ± 2.0 cm H_2O (13).

We did not observe any obvious limb edema in the sheep studies reported here. Nonetheless, if the network of newly regenerated lymphatic vessels increases resistance to lymph transport, a sub-clinical rise in interstitial fluid pressure may occur. This change in interstitial dynamics may prime the host for the future development of lymphedema because the threshold interstitial fluid pressure reflective of clinical edemas may be more easily achieved with additional insults to the system such as those caused by irradiation or infection.

ACKNOWLEDGMENTS

The authors wish to thank Ms. Dianna Armstrong for technical assistance. This research was funded by the Canadian Institutes of Health Research (CIHR).

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