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Lymphangiogenesis following obstruction of large postnodal lymphatics in sheep

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Abstract

We examined the impact of lymph flow obstruction in large post-nodal lymphatic vessels in sheep. A silk ligature was placed 2 cm downstream from the prescapular or popliteal lymph node and tightened to interrupt flow. At 6, 12 and 16 weeks after lymph flow blockage, a network of small interconnecting lymphatics (~10–40 μm in diameter) could be observed in the vicinity of the ligature. These were identified using antibodies to the lymphatic endothelial markers LYVE-1 or VEGFR-3 or unequivocally, with the upstream intraluminal injection of the non-specific cell dye CFDA-SE. The observed lymphangiogenesis coincided with increased levels of Prox1, Tie2 (Y992) phosphorylation, MAPK activation, and decreased Akt activition. In the popliteal preparations, saline was infused into the prenodal ducts upstream of the regeneration site. The slopes of the inflow pressure versus flow relationships were 17.3±3.6, immediately after vessel obstruction, 36.2±9.6 at 6 weeks and 15.0±5.3 at 12–16 weeks. For comparison, the average slope in a completely intact popliteal system was 3.1±0.3 (from a previous publication). The resistance to flow remained high up to 12–16 weeks after flow obstruction suggesting that normal flow parameters had not been achieved over this time. The lymph node appeared to have some role in limiting the impact of post-nodal lymph obstruction, a function that appeared to be compromised by lymph stasis.

Keywords: Lymphangiogenesis; Lymphatic vessels; Lymph nodes; Lymph flow obstruction

Introduction

Lymphangiogenesis is generally a vigorous process but there are many situations in which lymph flow is compromised after lymphatic injury. The lymphedema associated with cancer-related lymph node dissection is a good example (Armer et al., 2001; Rockson, 2001) and if left untreated, the edema can lead to recurring infections, impaired limb function, psychosocial problems and in extreme cases, malignant complications and life-threatening infections. There is considerable interest in reversing lymphatic dysfunction in lymphedema by applying appropriate molecular agents directly to affected tissues (Szuba et al., 2002) or by introducing the molecules through gene therapy approaches (Saaristo et al., 2002). While molecular therapies show theoretical promise, the enhancement of tissue drainage following pharmacologically induced lymphangiogenesis will provide numerous challenges. Many questions remain; in particular, we know very little about the physiological properties of the newly formed vessels and how the transport capabilities of regenerating ducts are integrated functionally into a lymphatic network that includes lymph nodes as well as pre- and post-nodal collectors.

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While the general tendency in lymphangiogenesis research is to remove a block of tissue and therefore, destroy many lymphatic vessels, there is some merit in simplifying the model such that only one vessel is injured or obstructed. When individual lymphatic trunks are severed, 'sprouts' emerge from the severed ends and with contributions from the surrounding areas, eventually re-establish flow (Gray, 1939). In this regard, lymphatic vessels in sheep may provide unique opportunities to study lymphangiogenesis since the collecting ducts are relatively large and can be manipulated individually. The objective of this study was to determine if the obstruction of large postnodal lymphatic vessels in sheep would induce a consistent lymphangiogenic response and if so, to investigate the impact of new vessel formation on lymph transport over time.

Materials and methods

A total of 40 sheep were used in this investigation. All experiments outlined in this paper have been approved by the ethics committee at Sunnybrook Health Sciences Centre and conform to the guidelines set by the Canadian Council on Animal Care and the Animals for Research Act of Ontario. Sheep were anesthetized initially by I.V. injection of sodium pentothal. Subsequently, 2.0–3.5% isofluorane was delivered through an endotracheal tube via a Narkomed 2 respirator for surgical maintenance. The surgical area was shaved and prepped with alcohol and betadine. Temgesic was given post surgically and as required thereafter to treat post-surgical pain. Antibiotic (Duplocillin) was administered I.M. 1 day prior to, and 2 days after surgery.

Surgical interruption of lymph flow

The lymphatics investigated in this report were large postnodal ducts and while the diameters of the vessels were quite variable, they approximated 1 mm for popliteals, 0.5–1.0 mm for prescapular and 1–2 mm for mesenteric vessels. To access the popliteal and prescapular vessels, an incision was made through the skin and the muscle was separated, exposing the node of interest. For access to the mesenteric lymphatic vessels, a laparotomy was performed and the mesenteric lymph node chain was observed to lie close to the ileal–cecal junction. To aid in the visualization of the post-nodal lymphatics, a 1% solution of Evans blue dye (in saline) was injected either into the subcapsular sinus of the upstream node or introduced in multiple sites into the drainage basin of the node. Within seconds the dye entered the postnodal ducts.

To obstruct lymph flow, a 2-0 silk ligature was placed around the post-nodal vessel at least 2 cm downstream from the node and the ligature tightened to interrupt lymph flow. In all cases, the surgical sites were closed and the animals returned to their holding pens. The sheep were sacrificed 6, 12 or 16 weeks after surgery.

Evans blue studies

In six sheep, the animals were anesthetized as outlined earlier and the site of obstruction was opened to visual inspection. Evans blue dye was injected into the subcapsular sinus of the upstream lymph node in an attempt to visualize newly formed vessels. The movement of dye past the ligature into the downstream postnodal segment was taken to indicate that some degree of fluid continuity had been restored.

Fluoroscopy

In 3 sheep (6 limbs) between 8 and 9 weeks post ligation, a mobile fluoroscopy system was used (BV Pulsera, Philips) to visualize the lymphatic vessels at various times post-ligation. An X-ray contrast medium (10–30 ml, Lipiodol, EZ-EM Canada, Therepex) was injected either into an upstream popliteal prenodal vessel or directed into the popliteal lymph node.

Immunohistochemistry

A total of 15 sheep were used to assess lymphangiogenesis using two immunofluorescence staining methods (intact preparations—3 animals, 6 weeks post ligation—6 animals, 12 weeks—4 animals, 16 weeks—2 animals). In one technique, the vessel endothelium was stained with the non-specific cell dye CFDA-SE (Molecular Probes # C-1157). This provided unequivocal identification of the lymphatics since the dye was introduced directly into the lumens of the vessels through injection into the subcapsular sinus of the upstream lymph node. 5(6)-CFDA SE [5 (and 6)-carboxyfluorescein diacetate, succinimidyl ester] (molecular weight 557.47) diffuses passively into cells and remains non-fluorescent until its acetate groups are cleaved by intracellular esterases. It fluoresces green. Additionally, we stained the sections with antibodies to LYVE-1 or VEGFR-3 (tagged with Cy3-red), molecular markers relatively specific for lymphatic endothelial cells.

Tissue blocks were removed surgically. The samples were embedded in Tissue-Tek O.C.T compound, placed in a base mold and frozen immediately at −80 °C. Sections (7 μm thick) were cut using a Leica cryostat model Leica CM 3050S-3-1-1 and were placed on glass microscope slides. The slides were washed 5 times in phosphate buffered saline (PBS—0.1 M) and blocked for 1 h at room temperature with 10% goat serum in phosphate buffered saline (PBS). After washing with PBS, the sections were incubated overnight at 4 °C with 1:50 or 1:100 dilutions of rabbit, anti-human LYVE-1 primary antibody (Research Diagnostics Inc) or similar dilutions of rabbit anti-human VEGFR-3 (Research Diagnostics Inc). The next day, the sections were washed with PBS and incubated with 1:100 dilutions of goat, anti-rabbit IgG antibody tagged with Cy3 (Jackson ImmunoResearch). In controls the primary antibody to the molecular markers was omitted. Finally, the slides were mounted in aquapolymount and coverslipped.

Immunofluorescence microscopy was performed with a Zeiss Axiovert 100 M laser scanning confocal microscope. The argon and helium/neon lasers were set to wavelengths of 488 and 543 nm for excitation of CFDA-SE and Cy3 respectively.

Molecular analysis

Tissues were harvested from 3, 6-week post ligation prescapular preparations for molecular analysis. Comparisons were made with tissues extracted in similar areas in non-ligated preparations. The samples were frozen immediately in liquid Nitrogen and stored at −80 °C before use.

Samples were homogenized in RIPA lysis buffer for 30 min on ice (10 mM NaH_2PO_4 pH7.5, 150 mM NaCl, 1% NP-40, 0.1% SDS, 1% Sodium Deoxycholate, 10 mM NaF, 2 mM EDTA, protease inhibitor cocktail; Complete-EDTA free, Roche USA), cleared by centrifugation and the supernatants collected for further analysis. Alternatively, tissues were placed in Trizol (GibcoBRL) and processed following the manufacturers protocol. In brief, the homogenized tissues and 200 μl of chloroform were added to 1 ml Trizol. Following centrifugation at 10,000×*g* for 15 min at 4 °C, the upper phase was removed and 300 μl of 100% ethanol was added per 1 ml of Trizol. After 5 min incubation at room temperature, DNA was isolated by centrifugation at 2000×*g* for 5 min at 4 °C. Proteins were then precipitated from the phenol–ethanol supernatant by 1.5 ml isopropyl alcohol per 1 ml Trizol. After 10 min incubation at room temperature, the protein precipitate was isolated at 12,000×*g* for 10 min at 4 °C. This precipitate was washed 3 times in 95% ethanol and resuspended in 1% SDS. Equal amounts of protein from the ligated and control tissues were separated by SDS-PAGE.

Proteins were transferred to polyvinylidene fluoride (PVDF, Perkin Ekmer) transfer membrane for immunoblotting. With the exception of phospho-Tie2 which was blocked in 7% BSA, all membranes were blocked in 3% milk/TBS for 60 min. The primary antibodies used for detection were phospho-Tie2 Y992 (Cell Signaling Technology USA); phospho-MAPK (Cell Signaling Technology USA, 9E10); phospho-Akt S473 (Cell Signaling Technology USA); Tie2 (Santa Cruz USA, C20); MAPK (Cell Signaling Technology USA); Akt (Cell Signaling Technology USA); Prox-1 (Upstate Biotech USA); and β-actin (Sigma USA). Proteins were visualized by enhanced chemiluminescence (Pierce USA).