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An Experimental Model for Lymphangiogenesis and Lymphatic Metastasis

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For patients diagnosed with cancer, metastasis is the most critical prognostic factor. Even after surgical removal and systemic chemo- or radiotherapy of the primary tumor, it is often the metastases that cause morbidity. One avenue of tumor cell metastasis is the circulation, but some of the most common cancers metastasize through the lymphatic system, as evidenced by the location of the metastases in local and/or regional lymph nodes or as in-transit metastases. Nonetheless, our understanding of lymphatic metastasis lags far behind that of the hematogenous route. One major block in illuminating the mechanisms of lymphatic metastasis is the lack of a cohesive understanding of lymphatic biology, function, and neogenesis. It is still unclear how tumors gain access to the lymphatics; they may alter peripheral lymphatic vessels, induce peripheral lymphangiogenesis, or simply invade preexisting lymphatics. The lack of a cohesive understanding of lymphatic biology is the major hurdle in studying lymphatic metastasis. Systematic studies can only be realized with the development of model systems of normal and tumor lymphangiogenesis.

In the previous funding year, with the generous support of the Department of Defense / CDMRP – BCRP, we successfully developed a unique *in situ* experimental model in the mouse tail skin for examining the process of lymphangiogenesis, and have also used that model to characterize the lymphangiogenic process. In fact, we have completed successfully all the specific aims of this project:

- (1) to determine an optimal collagen gel composition which will promote cell infiltration and attachment,
- (2) to establish long-term, *in vivo* cultures of the gels in the skin of the mouse tail,
- (3) to visually observe the system for water channels and/or lymphatic sprouts directly and noninvasively, and
- (4) to observe molecular and structural changes in the gel over time.

In Aim 1, we performed *in vitro* pilot studies and found that fibroblast proliferation and long-term viability was markedly better in type I rat tail collagen, extracted in our own lab, than in the commercially available "Vitrogen" type I bovine collagen. We also found that the rate of fibroblast infiltration into collagen gels was optimal at a collagen concentration of 0.2%. Finally, the addition of a variety of glycosaminoglycans (as we had proposed to test) did not noticeably alter the rate of fibroblast infiltration, proliferation, or viability. Therefore, we decided to use 0.2% type I rat tail collagen in the gels.

In Aim 2, we designed an optimal method for implanting the gels in a reproducible manner with excellent longevity. All procedures were approved by Northwestern University's Animal Care and Use Committee. The procedure is as follows. First, the mouse is anesthetized with a cocktail of ketamine/xylazine. Using a surgical microscope, a 2-mm long circumferential segment of skin is carefully removed from the midpoint of

the tail, exposing the core of bone, tendon, and blood vessels that remain intact. This effectively disrupts the lymphatic network without harming the larger arteries and veins that lie beneath the skin along the tail core. Left alone, this would cause chronic swelling of the tail since fluid clearance is disabled. We then place a transparent, gas-permeable silicone sleeve developed in our lab, custom fit to each tail before surgery to ensure a perfect fit, over the gap. Finally, we inject the 0.2% type I collagen into the gap, allow 20 minutes at 37°C to gel (via a heating pad), and gently wrap the tail with first aid tape (to avoid destruction by the mouse and to provide mechanical support).

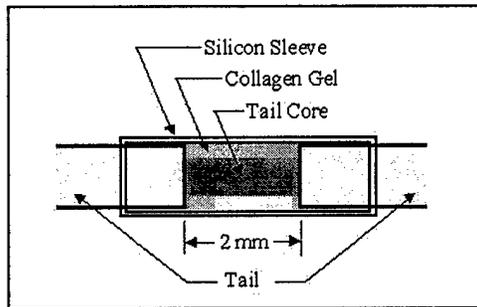


Figure 1. Schematic of the collagen construct in the mouse tail.

This collagen gel acts to restore fluid communication and provide a physical scaffold for ECM remodeling and cell migration. For fluid continuity, lymph fluid needs to travel through the gel in order to bridge the distal and proximal lymphatic vessels. We have maintained animals under these conditions for over 60 days; in fact, the gel becomes integrated with the existing skin within several weeks (see Fig. 2) and after this time the silicone sleeve is no longer needed and the tail appears perfectly normal. In control mice, 2-mm long by 1-mm wide segments of skin were removed (instead of complete circumferential excision), leaving intact lymphatics around the sides of the gel and therefore an alternative route for lymph flow. We did not see any relevant activity (vessel formation or

endothelial cell migration) in these gels.

In Aims 3 and 4, we sought to characterize the gel after lymphangiography revealed fluid continuity between the distal and proximal ends; the three evaluation techniques are shown

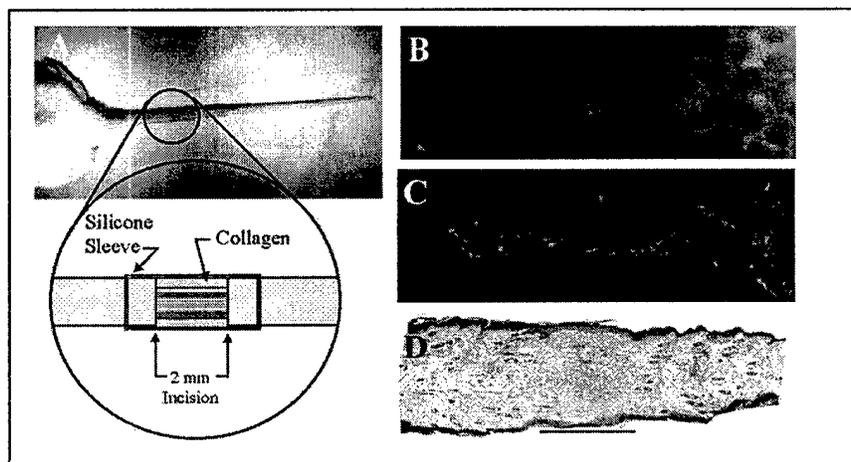


Fig 2. a. Schematic of collagen gel skin replacement model and photo of a mouse shortly after implantation. A 2-mm circumferential gap of skin is removed, leaving a core of bone, tendons, blood vessels, and nerves encased in a fascial layer. A silicone sleeve is fitted over the gap and a 0.2% type I collagen solution is injected and allowed to gel. **b.** Fluid channels through the gel that are continuous with lymphatic capillaries are made visible by fluorescence microlymphangiography (shown: day 25). **c.** Upon fixation, the FITC-dextran crosslinks to the tissue, permitting identification of those functional channels or lymph vessels in thin sections. Shown here is a section from the same mouse whose functional network is shown above in 5c. **d.** Standard hematoxylin and eosin staining of a thin section (again, of the same mouse tail) shows that within 25 days, the collagen gel appears to be well integrated with the existing tissue. The only way to identify the gel in sections is by the absence of hair follicles and epidermal glands. A normal epidermal layer, collagen architecture, and interstitial cell population can be seen within the gel, attesting to its biocompatibility. In all figures, distal end is on the right.

in Fig. 2. We have completed an evaluation of the gel and gel-tissue interface up through 60 days. Lymphatic function (*i.e.* fluid transport) and fluid channel architecture are evaluated *in situ* with fluorescence microlymphangiography (Fig. 2B), while histological and immunohisto-chemical techniques were used to assess ECM architecture, cell infiltration and organization, and cell phenotype. First, the collagen gel was well integrated with the existing tissue (Fig. 2D); the only difference we could detect between the gel and tissue was the fact that hair follicles and other glands were not present in the gel. A normal epidermal layer, collagen architecture, and interstitial cell population can be seen within the gel, attesting to its biocompatibility.

Fluorescence microlymphangiography is performed on the anesthetized mouse with lysine-fixable FITC-dextran (2000 kD) infused intradermally into the tip of the tail. Because of its high molecular weight, the solute is exclusively taken up by the initial lymphatics, revealing the functional lymphatic vessels and any fluid channels within the gel. Immediately following digital image acquisition, the mouse is perfusion-fixed and cryosectioned. During fixation, the dextran becomes crosslinked to the vessel wall so that

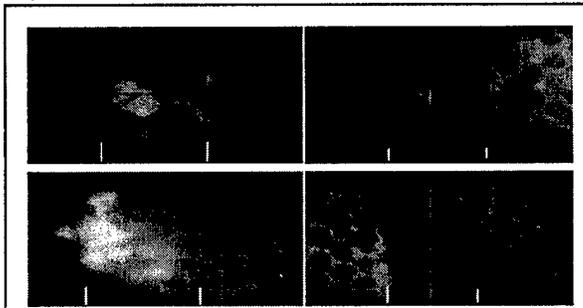


Fig. 3. Fluorescence microlymphangiography reveals the lymphatic vessels in intact skin and functional fluid channels within the gel, which may or may not be lymphatic channels (*i.e.* fluid channels lined with Flt-4 positive endothelial cells). Solid lines identify the location of the collagen gel (visible by eye). **a.** 3 days, **b.** 10 days, **c.** 25 days, and **d.** 60 days. Distal end is on the right.

any fluid channels and functional lymphatic vessels could be identified in the sections by the presence of FITC. Results are shown in Fig. 3. After 25 days, the lymphatic network was visible on the proximal side of the collagen gel following interstitial injection into the tail tip, implying that the gel construct

restores fluid communication between the distal and proximal lymphatics. By day 60, discrete fluid channels were clearly visible within the gel (Fig. 3d), and the hexagonal patterning is restored.

Even more interestingly, after sectioning, we found that many of the fluid channels were lined with endothelial cells by staining with an antibody against Flt-4, an endothelial receptor specific for lymphatic endothelial cells (Fig. 4). After 60 days (not shown), we saw a correlation of some FITC-dextran-filled fluid channels with positive staining for Flt-4. This confirmed the existence of lymphatic vessels within the gel.

Even more interestingly, after sectioning, we found that many of the



Figure 4. At 25 days, functional fluid channels, identified by the FITC-dextran tracer (shown in green), appear to form from the distal (right) end rather than the proximal (left) end. The same is true for LEC migration, identified with an antibody against Flt-4 (red). Cell nuclei are stained with DAPI (blue) and the location of the gel is marked by the white bars.

Implications

We had hypothesized that the physical stresses induced by interstitial fluid flow will initiate the (unknown) cascade of events that lead to fluid channel formation, cell migration and reorganization, and eventually, lymphangiogenesis. Using a unique modification of the mouse tail model, we showed that lymphangiogenesis can occur spontaneously in a tissue equivalent subjected to interstitial flow. We observed the formation of functional fluid channels that develop within 2-3 weeks within an initially acellular collagen gel. Furthermore, we confirmed that these fluid channels were lined with endothelial cells expressing Flt-4. Now that this is complete, we may begin comparing this process with that resulting from the presence of tumor cells in the gel in current and future studies. Clearly, last year's goals have been successful in establishing a novel and robust model in which to study tumor lymphangiogenesis. This work is currently under review for publication.