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Title: Validation of IRDye800 conjugated peptides imaging agent targeted to integrin α9 for optical imaging of lymphangiogenesis.

The lymphatic system is a major component of the circulatory system maintaining fluid and lipid homeostasis, as well as being the site for immune surveillance. Many diseases are associated with abnormal function of the lymphatic vasculature (such as obesity, asthma, cancer metastasis and lymphedema). Despite the importance of the lymphatics in disease little is known about the normal physiology of lymphatic vessels and the generation of new lymphatic vasculature, termed lymphangiogenesis. In the last ten years, markers for lymphatic endothelial cells (LECs) have been discovered, but the molecules involve in the process of lymphangiogenesis are still mostly unknown. The lack of understanding the lymphangiogenic process renders the understanding of its impact on human disease unknown. In my research project, I propose to validate the first marker for lymphangiogenesis and to develop an optical imaging agent to visualize lymphangiogenesis *in vivo.* A protein complex, integrin α9β1, has been linked to lymphangiogenesis in mouse models and *in vitro* experiments. Integrins are transmembrane proteins that provide a link between the cytoskeleton and the extracellular matrix. Such integrins are responsible cell anchoring to extracellular matrix, cell homing into tissues, motility of cells, and proliferation.[1] Integrin α9β1 is found in many different cell types including the LECs. Integrin α9β1 was found to bind to extracellular matrix components including fibronectin and vascular cell adhesion molecule-1 (VCAM-1) which are proteins express mostly during inflammation.[2] The link between integrin α9β1 and lymphangiogenesis in strengthened by the finding that mouse models of integrin α9 subunit knockout die within a week of birth due to lack of lymphatic remodeling, which cause lymph fluid to build up in the lungs.[3] Integrin α9β1 was also found to bind directly to three lymphangiogenic growth factors (i)VEGF-C, (ii) VEGF-D, and (iii) HGF.[4,5] Other facts that links integrin α9β1 to lymphangiogenesis are the increase expression of this integrin on lymphatic endothelial cells when in presence of the growth factors mention above. From these evidences, we believe that in integrin α9β1 is a key player in lymphangiogenesis and can be used as a marker for the process, see model figure 1. My hypothesis is that α9β1 integrin is a crucial signaling protein in lymphangiogenesis that regulates cell proliferation and migration, and can be used as a molecular target for optical imaging of lymphatic remodeling *in vivo* by using peptides containing a motif that binds specifically to the α9 subunit. The aims of my projects are (i) to validate that integrin α9 is a marker for lymph remodeling by identifying its role and pattern of expression during the process of lymphangiogenesis; (ii) to synthesize fluorescently-labeled peptides and to validate them as an imaging agent specific for α9 integrin; and (iii) to evaluate lymphangiogenesis in a mouse model of uncontrolled lymphangiogenesis and inflammation with the previously developed optical imaging agents.

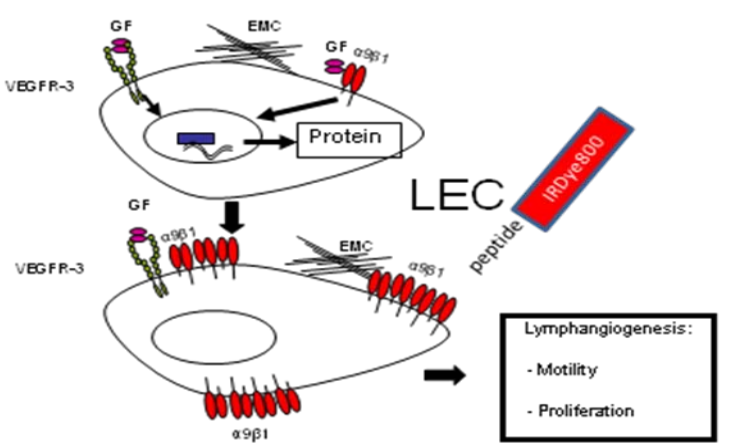

Figure 1: Our models is that upon binding of growth factor to the cell surface receptors, integrin α9 expression and affinity are increased. The cell signaling through integrin α9 promote the cell migration and proliferation, which in turn are required for lymphangiogenesis. If this model is true, integrin α9 can be used as a marker for the process of lymphangiogenesis.

For the first aim, I use both an *in vitro* and *in vivo* approach to determine the role of integrin α9 in lymphangiogenesis. I use a knockdown approach in cultured LECs to determine the effect of losing integrin α9 on cell motility and proliferation. I have identified two short hairpin RNAs (shRNAs) that can reduce the expression of integrin α9 in cell culture. In these experiments, I use the two validated shRNAs to decrease the expression of integrin α9 in the primary LECs from human origin. I test the ability of the cells to proliferate and migrate after the lost of integrin α9 expression. These studies allow me to determine if integrin α9 is required for these processes in cell culture. I also assess the role of integrin α9 *in vivo* by developing a tissue specific inducible knockout mouse for integrin α9, where administration of a drug induce the lost of integrin α9 function in mouse LECs. This mouse model is a unique approach to determine the specific role of α9 in the process of lymphangiogenesis during adulthood. These mice are used to test the role of integrin α9 in the production of lymphatic vessels by comparing the number of lymphatic vessels in the mouse after induction of lymphangiogenesis. We can induce lymphangiogenesis with administration of pro-lymphangiogenic growth factors and through chronic inflammation.[4,6] In addition to the knockout experiments, histology and flow cytometry are used to determine the expression level of integrin α9 on lymphatic vessels during lymphangiogenesis *in vivo*. I resect tissue from normal mice in which lymphangiogenesis was triggered, and perform analysis to compare expression of integrin α9 in the tissue undergoing lymphangiogenesis to the tissue in normal state.

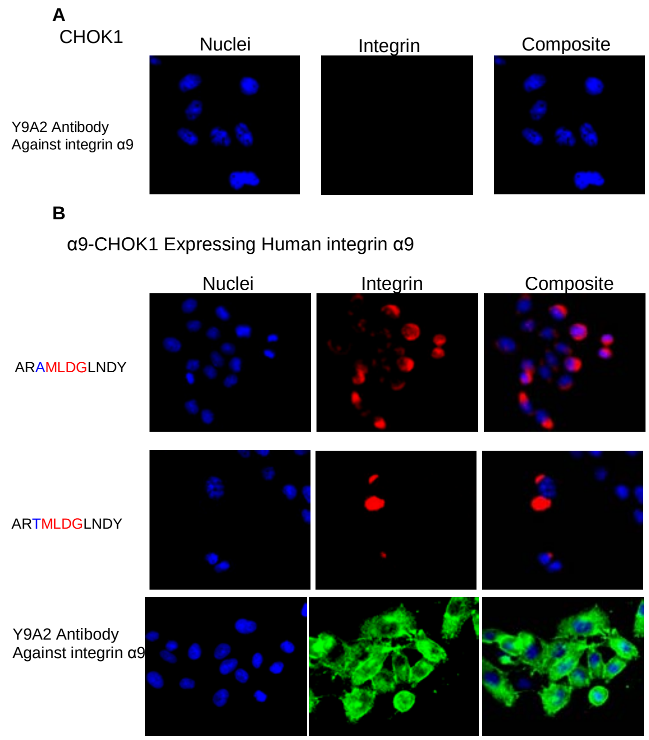

Figure 2: Cells were incubated with the MLDG peptides or the antibody Y9A2, against integrin α9, for one hour. Fluorescence microscopy was performed to assess the binding of (A-B) the antibody to CHOK1 cells or (B) the two peptide agents to α9-CHOK1 cells overexpressing human integrin α9. The two peptides bound to the α9-CHO clone, but the ARAMLDGLNDY shows higher fluorescence than the ARTMLDGLNDY.

Currently, two linear peptides labeled with IRDye800 (LI-COR Biosciences, Lincoln NE) were synthesized with preliminary results showing binding to CHOK1 cells over expressing human integrin α9, see figure 2. Additional peptides are being tested with specificity to integrin α9, see table 1. I further evaluated the specificity of the peptides with inhibition studies where binding of the peptides to integrin α9 in inhibited using a specific antibody which is known to inhibit binding on integrin α9 to its natural ligand, the vascular cell adhesion molecule-1 (VCAM-1) and fibronectin (the peptides selected were derived from the binding domains of these two ligand). Specificity of the peptides for integrin α9 is also assessed using knockdown experiments, where integrin α9 expression will be suppressed in cells expressing a variety of integrin and binding to the peptide will be determine. We have also performed binding assays for the two linear peptides which show an inhibition concentration (IC50) in the nano molar range.

Binding Motif	Peptide Sequence
EIDGIEL	GGGPLAEIDGIELA
EIDGIEL	GGGPLAEIDGIELTY
EIDGIEL	K(16)-GCPLAEIDGIELCA
MLDG	ARAMLDGLNDY
MLDG	ARTMLDGLNDY

Table 1: Sequences of peptides known to target integrin α9. The peptides will be synthesized and conjugated to IRDye 800 CW.

Specifically for the last aim, we will use mouse model of increased lymphangiogenesis. Two animal models are used to test the ability of the peptide imaging agents to recognize regions undergoing lymphangiogenesis in animal models. The first model is a transgenic knockout mouse which has RASA1 gene removed from the mouse genome. This mouse's phenotype shows uncontrolled lymphangiogenesis in the skin.[7] Preliminary data obtained by histological staining of RASA1 mice skin shows high expression of integrin α9 on their lymphatic vessels, see figure 3. I expect that our agent will be able to image the growing lymphatic in the skin of these mice. The second model we will be using is a model of skin inflammation. It was previously shown that an important step in inflammation is the formation of lymphatic vasculature in the inflamed area. Chronic inflammation will be induce in the mouse by applying a allergenic solution, oxazolone, onto the mouse skin.[6] We expect that the imaging agent will be able to specifically localize in the inflamed skin. All fluorescent imaging experiments are performed using the optical system developed in the laboratory of Dr. Eva Sevick.[8]

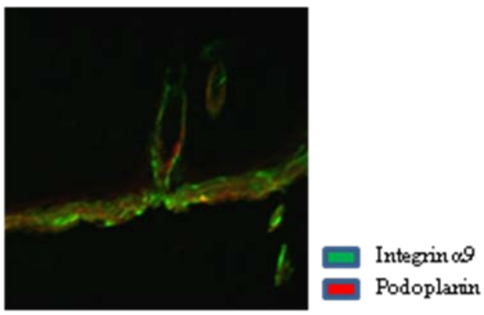

Figure 3: Histological staining (10X) of proliferating mouse skin lymphatic vessels with antibodies to integrin α9 and podoplanin (lymphatic marker). We can observe colocalization of the lymphatic marker podoplanin with integrin α9 on the lymphatic vessels. Further studies needs to be done to quantify the level of expression of the proteins.

If successful, our work could be used by researchers to further the understanding of the role of lymphangiogenesis in diseases states such as cancer, lymphedema, or arthritis and could potentially be translated into the clinic to diagnose lymphangiogenesis in patients suffering from these diseases that involve the lymphatic system. We would also provide the validation of the first marker for lymphangiogenesis, which could provide an understanding of the molecular mechanism of lymphangiogenesis.

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