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RESEARCH HIGHLIGHT

Watching lymphatic vessels grow by making them glow

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A novel imaging technique for visualizing the growth of lymphatic vessels in the cornea is summarized. Comparison to existing lymphatic imaging approaches and perspectives for future research are described.

Lymphatic vessels are critical for tissue homeostasis by draining excess fluid and macromolecules and for initiating an immune response. Lymphatic vessels also have key roles in a wide array of pathological conditions, including cancer, chronic inflammation, and transplant rejection. During the last 15 years there have been tremendous advances made in elucidating the major transcription factors and growth factors that are responsible for lymphatic vessel growth (lymphangiogenesis) and maintenance [1]. As work is progressing to translate these discoveries into therapeutic approaches that can either promote or impede lymphatic vessel growth, there is an existing need for improved *in vivo* imaging techniques that can visualize the remodeling of these vessels. The use of such methods in conjunction with established models of lymphangiogenesis would allow noninvasive monitoring of novel pharmaceutical interventions, as well as providing further insights into the molecular mechanisms taking place.

The murine corneal model is one of the most robust *in vivo* assays of lymphangiogenesis. It has several attractive features including a reproducible time course of sprouting lymphangiogenesis into a tissue not normally endowed with lymphatics, the ability to test growth factors or therapeutic compounds by implantation of slow release micropellets, and the capability to perform transplantation surgeries to test lymphatic involvement in the graft-rejection response [2]. Its value was recently demonstrated in a seminal study by Albuquerque and colleagues where a new endogenous inhibitor of lymphatic vessel formation was discovered [3]. As shown by the current study of Yuen and colleagues [4], the easily accessible location and transparent nature of the cornea make it ideal for *in vivo* fluorescent imaging.

The study describes novel techniques of video-rate stereomicroscopy and high resolution 2-photon microscopy to image the growth of the lymphatic vessels in the cornea after inflammation was induced by suture placement [4]. With injection of fluorescent dextran particles into the subconjunctival space, the normally invisible lymphatic vessels could be visualized. This method was used to reveal the changes in lymphatic morphology that take place during the time course of inflammatory lymphangiogenesis, including a rapid lymphatic vessel network formation and more gradual lymphatic regression. Importantly, the imaging could be reproducibly performed in the same animals over time, which may eliminate the requirement of sacrificing groups of mice at different stages for *ex vivo* analysis, potentially greatly streamlining preclinical studies of new therapies. To this effect, the authors demonstrated the potential of the technique to visualize the response to a known inhibitor of lymphangiogenesis, neutralizing antibodies targeting vascular endothelial growth factor 2 (VEGFR2). Although the effect on lymphatic vessel growth of diminished angiogenesis that would also be expected in response to VEGFR2 inhibition was not evaluated here, the simultaneous imaging approach described for blood and lymphatic vessels in the cornea could potentially be used in future to dissect the angiogenesis from the lymphangiogenic response to therapies. This is a key concern as many inhibitors or stimulators of lymphangiogenesis have effects on both vessel types.

Similar *in vivo* techniques using fluorescent tracers to visualize lymphatic vessels have been performed in other tissues, such as the mouse ear to image tumor lymphatics [5] and the tail for visualizing lymphangiogenesis during wound healing [6]. Unlike the corneal model, these models represent remodeling of an existing lymphatic network rather than sprouting into an alymphatic tissue, complicating interpretation of the detailed morphological changes that take place during lymphangiogenesis. Development of reporter mice

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with fluorescently-labeled lymphatics are currently a major research focus in lymphatic biology [7, 8]. While the current imaging techniques likely cannot be adapted to the *Prox1*-GFP mice that have been reported since they exhibit very bright fluorescence signals in the eyes and the *Vegfr3*-YFP phenotype for lymphatic vessels has not been completely described, additional mouse strains based on lymphatic specific genes and red fluorescent reporter genes will likely be developed in the near future. The use of transgenic reporter mice for visualization of the morphology of the developing vessels would eliminate the need for injections of tracers such as the fluorescein isothiocyanate-labeled dextran used in this study. Tracer injection, however, is a more suitable approach for patient condition when the technique is further developed for clinical application. With the use of additional existing fluorescent strains of mice, it may also be possible, as speculated by the authors, to visualize the interplay of lymphatics with fluorescently-labeled macrophages or dendritic cells.

Future research efforts should be aimed at using the imaging techniques to develop quantifications of morphology (density and caliber of lymphatic vessels, branching points, filopodia extensions *etc*.) that can be validated with existing immunohistochemistry approaches. Another key development would be assessments of the functionality of the remodeling lymphatics, perhaps by monitoring the clearance of the injected tracers from the inflamed corneal lymphatics over time. This is especially important in light of recent evidence that promotion of lymphatic function during chronic inflammation may help to resolve the condition [9]. While the transparent cornea has low autofluorescence and the use of tracers in the visible region are suitable for dynamic imaging of function, near-infrared imaging approaches are currently being developed that allow superior visualization of the pulsation of collecting lymphatics in other tissues such as the skin of mice [10, 11] as well as quantification of lymphatic flow through the sentinel nodes draining from tumors [12]. Due to the increased depth of the tissue that can be visualized with near-infrared wavelengths, dynamic fluorescent lymphangiography techniques are now being applied to the clinic [13].

The current paper represents an important technological advance in applying novel live imaging techniques to the corneal lymphangiogenesis assay for lymphatic vessel visualization. Although many additional advances must be made before *in vivo* imaging techniques can replace conventional *ex vivo* immunohistochemistry approaches, making previously unseen vessels glow in the living cornea represents an important first step.

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