Lymphangiogenesis: A Potential New Therapy for Lymphedema?

Running title: Cooke; 9cisRA and lymphangiogenesis

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The lymphatic circulation in health and disease

At the level of the capillaries, the systemic circulation loses about 2-4 liters of fluid and about 100g of protein into the interstitium daily. This ultrafiltrate of the systemic capillaries is returned to the circulatory system by the lymphatics. The lymphatic vasculature is highly specialized to perform this service, beginning with the blind-ended lymphatic capillaries. These vessels are highly permeable to protein, fluid and even cells, due to fenestrations in their basement membrane, and discontinuous button-like junctions rather than tight intercellular junctions as observed in the systemic capillaries. The lymphatic capillaries merge into collectors and larger lymphatic conduits that are invested with vascular smooth muscle (capable of contracting and propelling lymph forward) and valves for unidirectional flow. These conduits merge at lymph nodes, delivering antigens to the immune cells and serving as an early warning system of pathogen invasion. The lymph nodes drain into conduits that ultimately merge into the thoracic duct which empties into the left subclavian vein.

Lymphedema may be secondary to acquired obstruction of the lymphatics (as with surgery, tumor, trauma or infection). The most common cause of secondary lymphedema in developed countries is related to breast cancer and its treatment. In men, prostate cancer or lymphoma are common causes. By contrast, in primary lymphedema, the lymphatics are congenitally hypoplastic or maldeveloped. A number of mutations of genes involved in lymphatic development are associated with primary lymphedema (GJC2, FOXC2, CCBE1, VEGFR-3, PTPN14, GATA2 and SOX18). Developmental and genetic studies have shown that lymphangiogenesis requires the VEGF-C isoform of vascular endothelial growth factor, which binds to its receptors VEGFR2 and VEGFR3. The expression of these receptors on lymphatic
endothelial cells (LECs) during development is under the control of the transcriptional factor Prox-1, the master regulator of lymphangiogenesis\(^4\). This increased understanding of lymphangiogenesis has led to pre-clinical trials of VEGF-C augmentation that seem promising.

**A novel lymphangiogenic therapy?**

Accordingly, the manuscript by Choi and colleagues\(^5\) arrives at a propitious time. The authors provide evidence that 9-cis retinoic acid (9-cisRA) a naturally occurring retinoid that binds all known intracellular retinoic acid receptors\(^6\) is capable of inducing lymphangiogenesis in pre-clinical models. This is an intriguing observation as 9-cisRA is FDA approved for topical application in Kaposi’s sarcoma\(^7\); there is considerable clinical experience with this drug; and thus development and entry into clinical trials could be expedited. But before launching into our clinical trial, let’s take a closer look at the evidence.

To begin, Choi and coworkers observed that 9-cisRA and other retinoic acid derivatives increased the proliferation, migration and tubulogenesis of human LECs. This is surprising, given that 9-cisRA has been used to treat Kaposi’s sarcoma, a form of pathological endothelial proliferation, and that 9-cisRA and other retinoids have anti-proliferative effects\(^8,9\). It is worth noting that the investigators only explored a narrow range of concentrations with 9-cisRA (from 0.5-2\(\mu\)M). Is it possible that the investigators may have seen a biphasic effect of the agent, if higher doses were also studied?

In any event, the investigators hypothesized that these surprising effects of 9-cisRA might be mediated by angiogenic growth factors. Vascular endothelial growth factor (VEGF) or
fibroblast growth factor (FGF) are known endothelial mitogens, so it was reasonable to assess the effects of pharmacological antagonists of their action. Whereas two different antagonists of the FGF receptor blocked the effects of 9-cisRA, antagonists of the VEGFR-2 or VEGFR-3 receptors had no effect. Furthermore, LECs treated with 9-cisRA were observed to manifest a transiently increased expression of FGF receptors (FGFR3 and FRGR4). Finally, a soluble form of FGFR3 also blocked the effect of 9-cisRA on LEC proliferation (presumably by scavenging released FGF). So it seems reasonable to conclude that the effect of 9-cisRA, at least at lower doses, are mediated by FGF. This finding also raises a concern, discussed in more detail below.

In addition, Choi et al. observed that 9-cisRA downregulated the expression of two cell cycle inhibitors p27(CDKN1B) and p57 (CDKN1C), as well increased the expression of two aurora kinases whose activity promotes cell cycle. Further studies suggested that these effects of 9-cisRA could be mediated by non-genomic as well as genomic mechanisms. Specifically, the downregulation of p27 was due in part to its phosphorylation by Akt kinase (which is known to result in the degradation of p27). In contrast, the downregulation of p57 was on a genomic basis, mediated by the transcriptional factor Prox1 is known to upregulate p57. As expected, overexpression of Prox1 increased p57 expression, and downregulation (by siRNA) of Prox1 had the opposite effect. Exposure of the LECs to 9-cisRA caused Prox1 to dissociate from p57 (as detected by chromatin immunoprecipitation assay). Additional studies (including combined knockdown of Prox 1, and the nuclear receptor of retinoic acid RXRα) indicated that 9-cisRA is likely acting on additional cell cycle proteins, and that its effects may be mediated through other retinoic acid receptors other than RXRα. To conclude, 9-cisRA had non-genomic as well as
genomic effects on multiple cell cycle proteins, explaining in part its effects on lymphatic endothelial cells.

Finally, the investigators used four pre-clinical models to demonstrate the lymphangiogenic effect of 9-cisRA. After 5 days of intranasal application of 9-cisRA or vehicle, mouse trachea tissue manifested increased lymphatic vasculature. In a second model, matrigel was placed subcutaneously in mice, forming a plug that was harvested two weeks later. Those plugs which contained 9-cisRA displayed more prominent lymphatic capillaries, as detected by staining for the lymphatic marker podoplanin. Similar results were observed in the corneal micropocket assay. Most significant were their results with the mouse tail lymphedema model. In this model, a circumferential lesion is made at the base of the tail and the deep lymphatics severed, without damaging the systemic vessels. Intraperitoneal administration of 9-cisRA postoperatively reduced lymphedema, as assessed by tail diameter, and increased lymphatic capillary density, as assessed by immunohistochemistry. These findings were reproduced in a second strain of mice with a second set of investigators. So to conclude, in four pre-clinical models, 9-cisRA enhanced angiogenesis. These animal data were compelling, and it does appear that 9-cisRA, at the doses administered and over the time course of observation, had a lymphangiogenic effect.

**Time for a trial of 9-cisRA in lymphedema?**

I think not. A concern with any lymphangiogenic agent is the fact that for most patients in the U.S., cancer (or its attendant therapy) is the cause of the lymphatic obstruction. Because the lymphatics play an important role in metastasis, a lymphangiogenic agent in these patients would need to be used cautiously, with its administration limited in time and space.
There remains a concern regarding what effect the agent had on the systemic vasculature. Although 9-cisRA did not induce proliferation of human systemic (dermal) endothelial cells and there was no evidence of local angiogenesis when 9-cisRA was implanted in the mouse cornea, these studies of angiogenesis were not comprehensive. Nor did the investigators examine the effect of the agent on vasculogenesis (ie. mobilization of circulating endothelial progenitor cells). These data are needed, as it would not be useful to expand the systemic microvasculature, and increase blood flow, in the lyphedematous limb.

Furthermore, in some patients, any hydrodynamic solution for the swollen extremity will be insufficient. Although lymphedema begins as a lymphatic obstruction causing protein and fluid overload in the extremity, with chronicity of the condition, there may be dramatic tissue changes (not fully replicated in the mouse model). In this common progression of lymphedema, most of the dermal expansion is not due to fluid, but rather secondary to adipose tissue septated by bands of connective tissue, with inflammatory infiltrate\(^2\). This raises the question as to what might be the effects of 9-cis RA on the process of lipidogenesis, fibrosis and inflammation that occurs with established lymphedema, given that 9-cis RA activates a signaling pathway (ie. FGF) which is known to stimulate the growth of a diversity of cells\(^{10}\).

**What is the future for lymphedema therapy?**

Nevertheless, lymphangiogenic therapies may have a role. In a mouse model of inherited limb edema based upon mutations in VEGFR-3\(^{11}\) therapeutic overexpression of VEGF-C using a viral vector induces lymphangiogenesis and improvement in lymphedema. Similarly, in a rodent
model of surgically-induced lymphatic obstruction, the exogenous administration of human recombinant VEGF-C restores lymphatic flow\textsuperscript{12}.

Direct microsurgical anastomotic procedures have been used to restore lymphatic flow. Lymphovenous anastomoses can be made between lymphatic vessels distal to an obstruction in nearby small veins. This procedure allows lymph from the obstructed region to flow directly into the venous system\textsuperscript{13}. Normal autogenous lymphatic vessels have been used to bypass lymphatic obstruction. However, the long-term patency of these microsurgical approaches is minimal. It is possible that better results may be achieved using autologous lymph node implantation\textsuperscript{14}, particularly if combined with lymphangiogenic therapy.

**In the meantime, what is to be done to treat these patients?**

The investigators state that: “Lymphedema…. presents a considerable physical and social burden as there is not treatment currently available”. Perhaps the authors were referring to pharmacotherapy, which is certainly limited\textsuperscript{15}. Diuretics, although widely prescribed for this chronic, edematous condition, are rarely useful and may in fact be deleterious. Topical antifungals are useful, as mycotic infections frequently occur, and antibiotics (particularly those directed at staphylococcus and streptococcus) are invaluable for the acute cutaneous infections, to which these limbs are susceptible. However, chronic suppressive antibiotics have not been proven to prevent recurrences. Oral benzopyrone has its advocates, but a focused review of the available data concluded that the data were insufficient to support a recommendation\textsuperscript{16}. 
On the other hand, physiotherapy for lymphedema can be very beneficial. In particular, complex decongestive physiotherapy, which includes specialized massage (manual lymphatic drainage) and multi-layered bandaging, substantially reduces the girth of the extremity. Previously, we have shown that a reduction in limb volume of 40% can be achieved\textsuperscript{17}. These techniques can be combined with intermittent sequential pneumatic compression, which may augment the benefit\textsuperscript{18}. In addition, care should be taken to keep the skin well-hydrated with moisturizing creams, to avoid fissuring which would be a source for infection. Exercise should be encouraged, so long as the limb girth is maintained with a compressive garment. Water sports are particularly good for patients with lymphedema, because in this case the limb is supported by the hydrostatic pressure of the water.

For the patients with the chronic form of lymphedema characterized by adipogenesis, liposuction appears to be very useful\textsuperscript{19}. This surgical intervention is superior to the old reduction surgeries which could leave the patient with a painful scarred limb ravaged by recurrent infections. Physiotherapy should be continued to maintain the surgical gain.

To conclude, there is much that can be done to ameliorate lymphedema. More definitive therapies are under investigation, based on a better understanding of lymphatic development and lymphangiogenesis, as well as the pathological processes that contribute to, and which follow from, lymphatic obstruction.

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