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EDITORIAL

HYALURONAN AND LYMPHEDEMA

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The lymphatics play an important if rather unglamourous role in recycling interstitial fluid and dissolved macromolecules for the maintenance of tissue homeostasis. This role goes largely unnoticed during health, but becomes only too apparent when vessel blockage or destruction results in lymphedema. Among the macromolecules transported in lymph, the glycosaminoglycan hyaluronan (HA) – a large megadalton co-polymer of $(GlcNAc\beta1\text{-}4\ GlcUA)_n$ – may have particular significance in relation to the pathology of lymphedema, because of its capacity to bind water, support cell adhesion/migration, and induce tissue inflammation. Research into the biology of HA is well established in the fields of connective tissue, immune cell trafficking, development and cancer. Yet despite knowledge that HA is largely metabolized within the lymphatic system, this aspect of its biology has not been well investigated. The current issue of Lymphology contains two manuscripts of relevance to the field. In the first manuscript, Ningfei Liu (1) presents a comprehensive review of HA transport and metabolism in lymph vessels and lymph nodes respectively, and lists some evidence that suggests HA contributes to inflammation and fibrosis in lymphedema. The second manuscript, by Weber et al (2) relates to tissue engineering strategies for the potential treatment of lymphedema, and reports on efforts to use immobilized HA to influence the growth pattern of primary lymphatic endothelial cells in culture.

The characteristic feature of lymphedema is swelling of the limbs due to a failure of the lymphatic vasculature to drain lymph. The primary lymphedemas are rare congenital disorders caused in most cases (e.g., Milroy's disease) by autosomal dominant mutations in the kinase domain of *FLT4*, the gene that encodes the receptor for the lymphangiogenic growth factor VEGF-C (3). The much rarer lymphedema-distichiasis syndrome is caused by mutations in the forkhead DNA-binding domain of the winged helix transcription factor gene *FOXC2* (4). Other candidate genes for human disease identified from their phenotype in gene targeted mice include those coding for angiopoietin-2, a ligand for the TIE-2 receptor that regulates lymphatic vessel branch formation and permeability (5), podoplanin (6) and neuropilin-2, a receptor for the class III semaphorins that may also act as a VEGF/PlGF co-receptor on lymphatic endothelium (7).

The far more common secondary lymphedemas occur mainly as a side effect, for example, following radiotherapy, removal of axillary nodes for breast carcinoma ablation, or in the tropical disease filariasis. Regardless of the molecular defect responsible for disease, most researchers hold that the best strategy for future therapy is to redirect new lymphatic vessel growth in the affected

tissue using recombinant growth factors. This is now feasible with the identification of VEGF-C and its tyrosine kinase-linked receptor VEGFR3 as a major ligand/receptor pair promoting physiological lymphangiogenesis (8). Indeed, the delivery of recombinant VEGF-C using retroviral gene transfer has been shown to induce lymphatic vessel growth in skin (9), and similar approaches were recently shown to reduce the signs of fluid retention in an acute/subacute model of lymphedema in the rabbit ear (10,11). Whether the effectiveness of this therapy will successfully translate to the clinic remains to be demonstrated, and one must exercise caution given the disappointing results of early trials into therapeutic hemangiogenesis for ischemia. A further potential obstacle for therapeutic lymphangiogenesis of radiation/surgery-induced lymphedema is that administration of growth factor(s) to tissues containing residual tumor cells may carry the risk of promoting their spread to draining lymph nodes, in light of the finding that transgenic VEGF-C dramatically enhances nodal metastasis in xenotransplanted tumor models [see e.g., (12,13)]. Nevertheless, the prospect for growth factor-based treatment of lymphedema remains encouraging.

The tissue engineering approach to lymphedema therapy as alluded to in Weber et al (2) is altogether more speculative. The aim here is to re-direct lymphatic vessel growth in the edematous tissue by transplanting artificial HA scaffolds together with autologous lymphatic endothelial cells. In an earlier report, these workers also used three-dimensional HA scaffolds to maintain the native phenotype of autologous chondrocytes in culture, prior to their potential use for hyaline cartilage repair in osteoarthritis (14). The latest manuscript describes an *in vitro* system to study the directed growth and patterning of lymphatic endothelial cells on microstripes of HA immobilized on glass surfaces by photoactivated cross-linking. Because they are densely coated with the HA receptor LYVE-1, a type I transmembrane protein related to the leukocyte HA receptor CD44 (15), these cells might have been expected to adhere to the HA microstripes and form parallel arrangements of lymphatic endothelium. However, the opposite was the case, and the cells adhered instead to the HA-free surfaces on the amino-silanized glass slides to form linear arrays of endothelium. Interesting as these studies are, they raise a number of questions. Firstly, the lymphatic endothelial cells used by Weber et al (2) were isolated from thoracic duct tissue, rather than peripheral tissue capillary lymphatics, and it is not clear whether these cultures expressed the same LYVE-1 +, podoplanin +, VEGFR3+ phenotype as has been recently documented for small vessel lymphatic endothelium (16-18). Furthermore, it is becoming increasingly clear that lymphatic vessel endothelial cells do not sequester HA by default (Nightingale, T and DG Jackson, manuscript in preparation) and that appropriate activation may be required to unmask the HA-binding site on LYVE-1 – perhaps in a similar manner to the unmasking of HA-binding sites on CD44 during leukocyte homing (19). Secondly, conjugation of HA with the photoreactive 4-azidoaniline group used by Weber et al (2) may well have deleterious effects on receptor binding. For example, in our laboratory we have observed that over-conjugation of HA with fluorescein can completely abolish binding to LYVE-1 in transfected fibroblasts (15). Lastly, it should be remembered that HA is a highly flexible molecule and that its association with different binding proteins is likely to impose specific conformational changes that alter its physiological function (20). For example, HA attached to the surface of activated smooth muscle cells forms thick cable structures through interaction with the inter alpha proteinase inhibitor IαI (and possibly other HA-binding proteins) rather than forming a diffuse pericellular "coat" such as the ones seen around fibroblasts and chondrocytes (21). Moreover, it is to the HA cables rather than the coats that CD44+ leukocytes

preferentially adhere. It will be interesting to determine the effects of the various different HA-protein complexes on cultured lymphatic endothelial cells using structures that more closely resemble those of authentic extracellular matrices.

The use of artificial guidance structures to re-direct new vessel growth is undoubtedly an interesting idea. During normal vascular development for example, the lymphatic network is closely associated with the blood vasculature, and lymphatic trunks are frequently found wrapped around blood vessels. Indeed, the patterning of the arterial vessel network itself receives cues for guidance from the developing sensory nerves in skin (22). Although the molecular cues exchanged between networks are likely to be in the form of growth factors, it is also conceivable that the matrix surrounding one vessel type serves to direct the growth of the other.

Afferent lymphatic vessels are the major pipeline for the transport of HA to the lymph nodes during its degradative cycle (23), and therefore any interference in lymphatic circulation will have a direct impact on HA homeostasis. The article by Liu et al (1) explores the idea that the accumulation of HA *per se* may aggravate the symptoms of lymphedema. As discussed in her article, the level of HA in normal lymph is of the order of 10-20 µg/ml, and in interstitial fluid is in the range of 5-10 µg/ml. In lymphedema, this level rises beyond 50 µg/ml, and in post-mastectomy edema, the concentration of accumulated HA (estimated as total non-sulphated soluble glycosaminoglycan) may approach 200 µg/ml (24). However, even a 200 µg/ml solution of HA would exert an osmotic pressure of only 5mm H_2O (24), and it is therefore unlikely that the levels of HA encountered in lymphedema could make a significant contribution to fluid retention through "osmotic drag." However, if accumulation of HA in edematous tissue becomes immobilized – for example through binding extracellular matrix components in the interstitia or cell surface HA receptors, then the true levels may be large and the additional water of hydration could contribute to tissue swelling. Further studies will be needed to determine whether accumulated HA is indeed immobilized in edematous tissue.

Lymphedema is more than just a defect in lymph vessel function. The condition has many aspects of an inflammatory disease, and in common with chronic inflammation, is accompanied by tissue fibrosis. The high incidence of bacterial infection in lymphedematous tissue may be one cause of episodic inflammation; alternatively, a component or components of lymph fluid could be responsible for both inflammation and fibrosis, and a clear candidate is HA itself, as suggested by Liu (1). This is certainly an appealing hypothesis, since oligosaccharide breakdown products of HA have pro-inflammatory properties *in vitro,* including the capacity to induce the release of chemokines and cytokines from macrophages (25,26), and significant levels of HA fragments almost certainly accumulate in lymphedema fluid. It is perhaps significant that in another context, that of lung injury, the accumulation of HA fragments induced by targeted CD44 gene deletion is also associated with prolonged inflammation and increased tissue fibrosis (27). The waste management of biologically active matrix products may well be a function of lymphatic vessels and could explain why terminal hydrolysis of molecules such as HA occurs in distant lymph nodes (23). Should further research confirm the relationship between HA and tissue pathology in lymphedema, then the enzymatic pathways for HA degradation may become new targets for therapy.

Loss of an afferent lymphatic network and stagnation of tissue fluid are believed to contribute to the well-documented increase in incidence of infections in lymphedema by blocking recruitment of the cellular immune response. Whether in normal tissue HA is involved in this process is an interesting question. Certainly there is good evidence to

support a role for HA and its primary receptor CD44 in the extravasation of leukocytes across inflamed blood vessel endothelium [reviewed in (28)]. Could the trafficking of antigen presenting cells from tissues to lymph nodes depend upon similar interactions as suggested by Liu? As already stated, lymphatic endothelium is coated with the HA receptor LYVE-1, a molecule that could in principle promote adhesive interactions with CD44+ dendritic cells *via* mutual binding to soluble HA molecules (29). In a scenario that reiterates our own earlier hypothesis, this arrangement could facilitate the intravasation of afferent lymphatics by antigen presenting cells (29,30). However, as outlined above, the regulation of LYVE-1 function may be quite complex, and these issues may only be resolved by experiments on mice with targeted deletion of the LYVE-1 gene. Whatever happens, the future is likely to be interesting for the field of lymphatic HA biology.

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