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Review Article

Chronic lower limb wounds evoke systemic response of the lymphatic (immune) system

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ABSTRACT

Wound healing should not be considered as a process limited only to the damaged tissues. It is always accompanied by an intensive local immune response and in advanced stages, the systemic lymphatic (immune) structure. In this review we present evidence from our own studies as well as pertinent literature on the role of skin and subcutaneous tissue lymphatics at the wound site and of transport of antigens along with collecting afferent lymphatics to the lymph nodes. We also speculate the role of lymph nodes in raising cohorts of bacterial and own tissue antigen-specific lymphocytes and their participation in healing and not infrequently evoking uncontrolled chronic immune reaction causing a delay of healing. It is also speculated as to why there is a rapid response of lymph node cells to microbial antigens and tolerance to damaged-tissue-derived antigens occurs

KEY WORDS

Healing; immunity; wound

INTRODUCTION

Wound healing should not be considered as a process limited to damaged tissues only. It is always accompanied by an intensive response of the regional and, in advanced stages, the whole body's lymphatic (immune) system. Penetration of microorganisms and cellular changes caused by tissue injury are almost immediately recognized by the local lymphatic system irrespective of the topography of tissue. Blood immune cells and plasma humoral factors extravasate through the process of chemotaxis and increased capillary permeability. The migrating immune cells ingest the microbial antigens as well as self-antigens from the apoptotic disintegrated tissue parenchymal cells and thence migrate via the initial and collecting lymphatics to the regional lymph nodes. There the elimination of antigens and raising of antigen-specific lymphocytes take place.

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The lymphatic system is a widespread vascular network that plays a vital role in homeostasis of the extracellular space. The role of the lymphatics is often neglected and the aim of this review is to emphasize the important contribution that the lymphatics make towards the maintenance of cell equilibrium and normal wound healing. The most important role of the lymphatics is the control of the interstitial fluid microcirculation. The lymphatic vessels removed from the extravascular space macromolecules and particulate matter is too large

to re-enter the blood capillaries. If these materials are not removed, the osmotic and hydrostatic forces within the tissues change and disease results.[1-4] Failure of the lymphatics leads to pollution of tissues by the excess protein, other macromolecules and fluid around the cells, as well as debris from wounds and microbes.[5,6] It is known that patients with lymphedema are prone to develop secondary infection as the lymphatics are a normal pathway for clearance of bacteria from the interstitium.

This review speculates the role in wound healing of the skin and subcutaneous tissue lymphatics at the wound site and also of transportation of antigens along collecting afferent/lymphatics to lymph nodes. There are several unanswered questions, e.g. what is the role of lymph nodes in raising cohorts of bacteria and own tissue antigen-specific lymphocytes and their participation in the healing process. There are questions related to the local autoimmune reaction. And finally, speculation on the rapid response of lymph node cells to microbial antigens with simultaneous tolerance to damaged-tissue-derived antigens.

BACTERIAL FLORA OF LIMBS COLONIZING WOUNDS

Human skin harbors a complex microbial ecosystem, with transient, short-term as well as long-term resident biota, based on the consistency with which they are isolated. *Staphylococcus*, *Micrococcus*, *Corynebacterium*, *Brevibacteria*, *Propionibacteria and Acinetobacter* species are, among others, regularly cultivated from normal skin. *Staphylococcus aureus*, *Streptococcus pyogenes* and *Pseudomonas aeruginosa* may be transient colonizers, especially in pathological conditions.[7-9] Aerobic bacteria were isolated from gap callus of 14% healing and 35% non-healing closed fractures. No isolates were found in subcutis and only in 3% in muscles. No anaerobic bacteria were detected. Polymerase chain reaction amplifications of 16 S rRNA were found positive in 42% of callus specimens proving the presence of bacterial DNA even when no isolates were found. The 95% similarity of the genetic pattern of some strains from foot skin and callus, estimated with random amplification of the polymorphic DNA technique, suggested their foot skin origin.[10] Among ischaemic limbs, bacterial cells were found in 58.6% specimens of tibial and popliteal vascular bundles and similarly, in 33.8% of femoral bundles. In the control group of healthy individuals, among femoral vascular bundle specimens, microbial cells were isolated in 11% ($P < 0.05$). Lower limb lymphatics of patients with fractures contained bacterial cells in 76%, as against 10% which controls. Majority of the isolates in limb arteries belonged to coagulase-negative staphylococci and *S. aureus*. There were also other highly pathogenic bacteria namely *Enterococcus, Proteus, Pseudomonas, Micrococcus, Klebsiella, Enterobacter, Serratia, Acinetobacter and Citrobacter.*[11] A high prevalence of bacterial isolates from the tissue fluid (64%), lymph (75%) and inguinal lymph nodes (66%) of limbs with filarial lymphedema has been found with *Bacillus cereus, Staphylococcus epidermidis, S. hominis, S. capitis, S. xylosus* and *Micrococcus spp*. being the most common isolates. Bacterial strains of the same phenotype and antibiotic sensitivity were documented on the toe web surface and in tissue fluid (25%), lymph (26%) or lymph nodes (41%).[12]

LOCAL IMMUNE EVENTS IN THE WOUND AND THE ROLE OF TISSUE DRAINING LYMPHATICS

Wounding of epidermis causes penetration of the body's own surface microbes - most commonly *S. epidermidis*, other coagulase-negatives and *Corynebacteria* - as well as activation of keratinocytes. Stimulation of keratinocytes upregulates their production of chemokines and cytokines [Figure 1]. These attract granulocytes, monocytes, tissue macrophages and dendritic cells. Also they stimulate proliferation of basal keratinocytes. Among the immune migrating dendritic cells, the most active are epidermal Langerhans' cells.

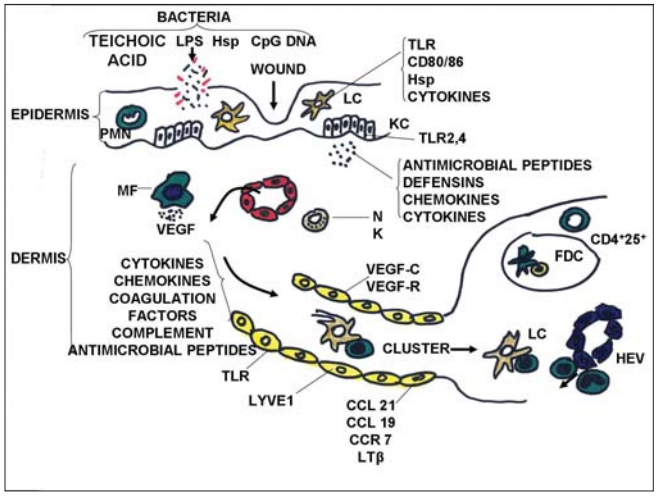

Figure 1: Immune events in wound or infection of epidermis. Keratinocytes, Langerhans' cells, macrophages, lymphocytes, endothelial cells, lymphatic endothelial cells and lymph nodes become activated and plethora of cytokines and chemokines is produced. Information is transferred via lymphatics to lymph nodes

Langerhan's cells absorb foreign and tissue antigens and migrate to the initial lymphatics and further with lymph stream to the lymph nodes where the processed antigen is presented to lymphocytes. Already in the flowing lymph, they attract lymphocytes and form rosettes.[13] They transfer the processed antigen to lymphocytes. In the wounded dermis extravasation of lymphocytes and precursors of dendritic cells takes place. They are an additional source of cytokines,which is regulating the healing process by stimulating fibroblasts. All locally produced and plasma filtered cytokines flow towards the initial lymphatics and are then transported to lymph nodes [Figure 1]. Concentration in lymph is always higher than in serum. The lymphatic endothelial cells produce chemokines, which attractlymphocytes like CCL19 and CCL21 for T cells and CXCL 13 for B cells, the absence of which assists directional migration to lymphatics. Taken together, blood supplies wounded tissue with immune cells and proteins whereas lymphatics absorbs free and cell-bound antigens and transport them to lymph nodes for the formation of antigen-specific cytotoxic and phagocytic cells. These cells most likely return via the blood circulation back to the wound to home there.

However, for antigens to enter lymphatics, lymphatics should be patent and be functional fluid conduits. Lymphatics behaviour depends on the type of injury. In acute penetration of microbes and subsequent inflammation of the skin they remain blocked for some days in the 'no flow' process preventing spread of noxious factors. Later in the traumatic or surgical wounds, they regenerate at the capillary level.

Lymphatics are relatively resistant to mechanical trauma. Trauma equal to 50% of the minimal energy[14] needed for tibia fracture (3.7 joules/g) was applied to the leg of hairless mice. Lymphatics were stained with fluorescein isothiocyanate-dextran injected into the footpad. They remained patent, with faster visualization and increased the average cross-sectional area in traumatized extremities as well as increased the lymph formation and flow rate.[14]

Regeneration or growth of lymphatics in and around wounds is up-regulated through cytokines and growth factors: the intrinsic relationship between lymphatic endothelial cells (LECs) and extracellular matrix microenvironment (ECM). ECM molecules and remodelling events play a key role in regulating lymphangiogenesis. Molecules related to 'functionality', especially hyaluronan, integrins, reelin, IL-7 and matrix metalloproteinases, provide the most fundamental and critical information prerequisite for LEC growth, migration, tube formation and survival, although lymphangiogenesis is directly as well as indirectly controlled by VEGF-C/-D/VEGFR-3-Prox-1-(vascular endothelial growth factor and receptor), Syk/SLP76-, podoplanin/Ang-2/Nrp-2-, FOXC2- and other signalling pathways in embryonic and pathological processes.[15] VEGF-A promotes lymphatic vasculature formation via activation of VEGFR-2 on lymphatic endothelium and lineage-specific differences of integrin receptor expression contribute to the distinct dynamics of wound associated with angiogenesis and lymphangiogenesis.[16] Lymphatic growth is regulated by VEGF C and its cellular receptor VEGFR-3. VEGFR-3-positive vessels were observed in the granulation tissue from day 5 onwards. Unlike blood vessels, very few VEGFR-3-positive lymphatic vessels persisted on day 9 after injury, and none were found on day 14. In chronic wounds such as ulcers and decubitus wounds of the lower extremity of humans, VEGFR-3 was also weakly expressed in the vascular endothelium. These results suggest that transient lymphangiogenesis occurs alongside blood angiogenesis and helps in healing wounds.[17] Between days 7 and 15 of injury, VEGF-C-induced lymphangiogenesis occured in both the subcutaneous tissue as well as dermis along the wound-healing edge, especially in the transitional area between the two, which is in any case favourable to growth of regenerating lymphatic vessels.[18] Lymphatic regeneration after replantation of the operated hind limbs of rats occurs by the 7th and 11th postoperative day. This has been confirmed by indirect lymphangiography and clinical observation of the post-traumatic lymphoedema. The average time of visualization of lymphatic regeneration through lymphography was 10–12 days. To achieve the best lymphatic drainage and the ability to use the replanted extremities, it is important to resect all non-vital tissues of the replantation area. Local or general infections decelerated lymphatic regeneration.[19]

Various pathological conditions are associated with delay in lymphangiogenesis around the wound. LYVE-1-positive lymphatic vessels and CD31-positive blood vessels were significantly reduced in corneal wound healing in diabetic mice (db/db) ($P < 0.02$) compared with control (db/+) mice. Glucose treatment of control macrophages led to the down-regulation of the lymphatic-specific receptor VEGFR3 and its ligaments, vascular endothelial growth factor-C and -D (VEGF-C, -D).[20] Podoplanin is a protein which is of specific importance among others

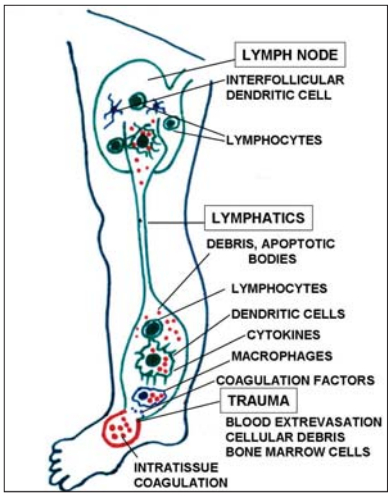

Figure 2: The pathway for information of damaged skin and deeper tissues by trauma or infection leading to lymph nodes

to newly formed lymphatics. The recovery of lymphatic vessels using podoplanin immunohistochemistry in the rat skin incision wound reveal that subcutaneous tissue of the incised skin area did not show any recovery of lymphatic vessels up to 84 days after the skin incision.[21] Recent studies suggest that chronic T-helper cell (CD4+) inflammation may contribute to fibrosis and lymphatic dysfunction in chronic lymphedema. The absence of T-cell-mediated inflammation markedly decreases tail edema and accelerates lymphatic regeneration during wound healing. Systemic depletion of T-cells markedly decreased TGF-beta expression in tail tissues. Inhibition of TGF-beta function promoted lymphatic regeneration, decreased tissue fibrosis, decreased chronic inflammation and Th2 cell migration, and improved lymphatic function.[22] Hypoxia inducible factor-α (HIF-1 α) is the central regulator of lymphangiogenesis. HIF-1α inhibition by small molecule inhibitors (YC-1 and 2-methyoxyestradiol) resulted in delayed lymphatic repair, decreased local vascular endothelial growth factor-C (VEGF-C) expression, reduced numbers of VEGF-C cells and reductions in inflammatory lymphangiogenesis.[23]

CHANGES IN MAJOR LYMPHATICS AND NODES IN LIMB WOUNDS

Traumatized, infected or inflamed tissue as well as necrotic ulcer are prone to colonization by microbes and release of self-antigens following the pathological change. Lymphatics drain these sites and transport information material to the regional lymph nodes. [Figure 2] The resultant events in the lymphoid tissue remain clinically unrecognized. There are usually no enlarged palpable nodes in the groin. Lymphoscintigraphic imaging of lymphatic pathways and nodes revealed the phenomenon of a major clinically silent reaction of the lymphatic system to these pathological developments in the tissues. Mechanical injury to soft tissue and bones of lower extremities is frequently followed by long-lasting edema at the site of trauma and also further distally. Interruption of lymphatics is considered to be the main etiologic factor. We suggest that protracted healing of injured tissues and bones with secondary involvement of the regional lymphatics and nodes may be responsible for the persistence of edema. Stimulation of the lymphatic system during the first (scavenging) phase of healing of traumatized tissues follows events such as hematoma, extravasation of bone marrow cells to soft tissues and colonization by microorganisms.

Extravasated blood does not produce changes in the skin, subcutaneous tissue or lymphatics; however, it does stimulate lymph node lymphocytes. Bone marrow cells and saprophytic bacteria cause a major local and lymph nodal inflammatory response.[24] Evaluation of the immune and lymphatic system response in trauma patients having closed lower limb fractures and soft tissue injuries was done by isotope lymphography. Dilated lymphatics of the entire limb were found in all, with 62% of them showing enlarged inguinal lymph nodes. [Figure 3] Venous thrombosis was found in only 24% of cases.[25] Interestingly, a decrease in the size occurredin the inguinal lymph nodes alongwith a dilatation of the deep lymphatics. Enlargement of popliteal nodes was seen in a majority of patients with non-healing fractures. The last event was most likely related to necrosis and depletion of inguinal lymph node cells by toxic factors absorbed from the non-healing wound.[26] Open wounds as well as non-healing ulcers also cause a reaction inthe lymphatic system. Lymphoscintigrams of the affected limbs show dilated lymphatics due to high transport needs related to the excess of tissue fluid/lymph produced in inflamed tissues [Figures 4, 5].

WHAT IS THE ROLE OF THE LYMPHATIC SYSTEM IN WOUND HEALING (AND NON-HEALING)

Intensive transport of microbial and self-antigens along the lymphatics to the lymph nodes and their cellular reaction in the lymphoid tissue results in the formation of antigen-specific cohorts of cytotoxic lymphocytes. It

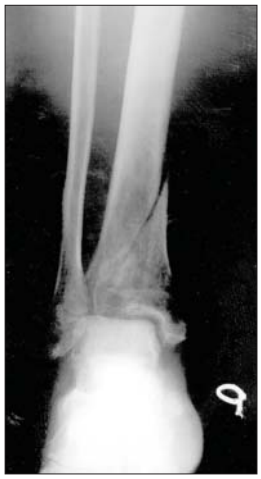

Figure 3: Left: fractured tibia. Right: lymphoscintigram depicting dilated lymphatics and enlarged inguinal lymph nodes in the same limb

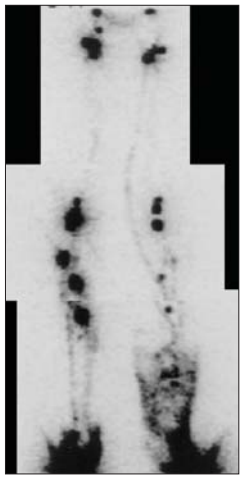

Figure 4: Lymphoscintigram of lower limbs months after mosquito bites. Swollen left leg with spread of isotope in the skin and subcutaneous tissue. Visualized popliteal lymph nodes. In the right limb, enlarged popliteal and inguinal nodes

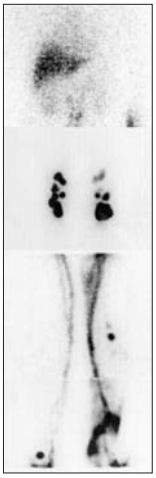

Figure 5: Lymphoscintigram in left leg venous ulcer. Enlarged lymphatics and inguinal nodes

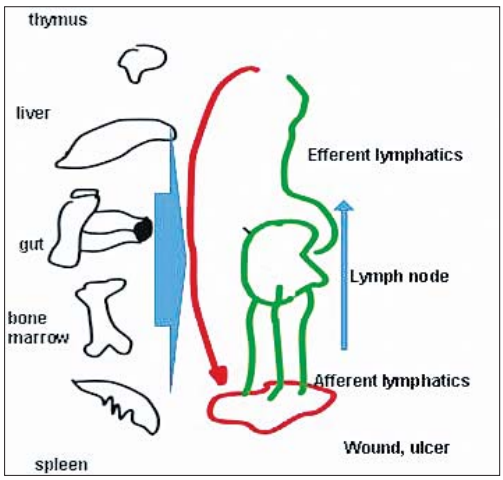

Figure 6: Hypothetical loop 'wound–afferent lymphatics–lymph node–efferent lymphatics–blood–wound'. Explanation in the text

remains so far unknown whether these cells migrate back through the blood stream to the wound, home there and if so, whether they participate in the healing process. The effect of homing lymphocytes may be pro- and anti-inflammatory as well as pro- and anti-lymphangiogenic. Lymph nodes are breeding sites for quick reaction to bacteria targetting their elimination. They may also be the sites for raising tolerance to self-antigens created from wound cellular debris. One reason for delayed wound healing could be thatthis low level of tolerance is insufficient in overcoming an excessive mass of self-antigens. Possibly in non-healing wounds, the aggressive lymph node-derived cells prevent healing by attacking their own granulation cells. Around 20% of long-lasting venous ulcers are complicated by systemic allergic reactions. An open question remains that is whether there is a closed functional loop between 'wound–regional lymph node and the blood circulation–wound', and what may be the tasks of the lymphocytes and precursors of dendritic cells circulating in this loop. [Figure 6] The hypothetical loop 'wound-afferent lymphatics-lymph node-efferent lymphatics-blood-wound': antigens are transported from wound via afferent lymphatics to lymph node where antigen processing takes place followed by proliferation of antigen-specific lymphocytes; these newly formed cells are transported along with the efferent lymphatic via the thoracic duct to blood circulation; some of them are trapped in the liver, gut, bone marrow and spleen and inform local lymphoid tissue about penetration of the body by microbes and release of own cellular debris. These antigen-specific cells are further extracted from blood at the wound site; there they participate in the healing and reconstruction processes; however, they may also attack their own granulation cells as a form

of an auto-immune reaction. Debris promotes bacterial colonization. This may explain the delay in wound healing and systemic allergic reaction seen in some 20% patients with long-lasting wounds.

And a final question is: with raising of antigen-specific memory and of Treg cells in the nodes,would there be less reaction in the lymph node to the microbial and tissue antigens and whether it would result in the faster healing of secondary wounds?

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