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Evaluation of lymphatic regeneration in rat incisional wound healing and its use in wound age estimation

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Wound age;
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Abstract *Objective:* During the wound healing process, lymphatic regeneration in the injured skin has not been fully investigated. This work was designed to study the regeneration of lymphatic vessels in rat incisional wounds in relation to the duration after the wound infliction.
Material and methods: We studied the regeneration of lymphatic vessels in the rat skin incisional wounds (sutured and unsutured) by immunohistochemistry using an antibody against D2-40, a marker of lymphatic endothelium.
Results: Lymphatic vessels were detectable transiently at the wound edge and depth from day 3 till day 7, and none on day 10 in sutured wounds; and from day 5 till day 10, and none on day 14 in unsutured wounds. On the other hand, the center of the wound area did not show any evidence of lymphatic regeneration up to 60 days after the skin incision, regardless of presence/absence of sutures. Meanwhile, the regenerating blood vessels started to appear in the granulation tissue as early as day 2 in sutured wounds and day 3 in unsutured wounds.
Conclusion: Lymphatic elements appear transiently in the wound edge, concurrent with the appearance of blood vessels but regress earlier. Identification of lymphatic vascular channels in the region of the wound may help to estimate the wound age in the early days after the injury. At later time points in the regeneration process, it may help to recognize the injured area, being the area where the dermis and subcutaneous tissue are devoid of lymphatics.

Abbreviations: PBS, phosphate-buffered saline; DAB, diaminobenzidine

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1. Introduction

Wound healing is a dynamic process that proceeds with well-organized interaction between soluble mediators, blood cells, extracellular matrix, and parenchymal cells. Cutaneous wound healing starts immediately after injury and consists of three sequential phases: inflammation, proliferation, and maturation.[1] The initial injury causes platelet adhesion and aggregation, and the formation of a clot in the surface of the wound, leading to inflammation. In the proliferative phase, there is formation of granulation tissue, proliferation and migration of connective tissue cells, and re-epithelialization of the wound surface. Maturation involves extracellular matrix deposition, tissue remodeling, and wound contraction.[2] In each phase, various kinds of biological substances are closely involved. Examination of the dynamics of such a process would be beneficial to find a clue for wound vitality or wound age.[3]

Angiogenesis, the development of new blood vessels from pre-existing ones, is a crucial event in the formation of granulation tissue in the proliferative phase of wound healing.[4] This process has been studied extensively. Although lymphatic vessels are also present in the subcutaneous tissue, lymphatic regeneration in injured tissues has not been fully investigated.[5]

Lymphangiogenesis, like angiogenesis, is known to occur through sequential steps that involve production of vascular endothelial growth factor, expression of matrix metalloproteinases; and endothelial cell migration, proliferation and organization into functional vessels.[6] Although many similarities exist at the cellular level, the organizational principles of blood and lymph angiogenesis are different, and are probably related to their distinct physiological functions; angiogenesis is a tissue response to hypoxia[4] whereas lymphangiogenesis is a tissue response to interstitial fluid flow.[7]

Lymphangiogenesis has been reported to occur in adult tissues during inflammation, wound healing, and tumor metastasis.[8] Earlier studies have demonstrated at least some lymphatic vessels sprouting in experimental rabbit ear wounds.[9]The growth of new lymphatic vessels has also been detected in autotransplants of the rat small bowel[10] and of the rat hindleg.[11] Increasingly, the importance of lymphatic biology is being realized. However, to date, lymphangiogenesis in adult tissues is not adequately understood,[6] and studies addressing lymphatic regeneration in the wound healing process remain insufficient, and have yielded contradictory results.

The recent identification of lymphatic endothelial markers has enabled the specific identification of lymphatic vessels.[12,13] D2-40 is a commercially available monoclonal antibody that specifically detects a fixation-resistant epitope on podoplanin, which is a mucin-type transmembrane glycoprotein that is specifically expressed by lymphatic endothelial cells,[14–16] but not by vascular endothelial cells.[17]

The aim of the present study was to investigate lymphatic regeneration in a rat incisional wound model along the period of the wound healing process in order to determine the age of the wound to be used in forensic applications.

2. Material and methods

2.1. Experimental design

A total of 66 albino rats (6 week old, 150–200 g) obtained from the animal house of the Medical Research Institute, Alexandria University, were used in the study. The animals were allowed free access to a standard pellet diet and water, and were maintained at 21–23 °C in a 12/12 h light/dark cycle. All procedures complied with the guide lines for the care and handling of animals, and the study protocol was approved by the ethics committee of Alexandria Faculty of Medicine.

2.2. Skin wound model

All wounding procedures were carried out under inhalational diethyl ether anesthesia. After shaving the hair of the dorsal thigh on both sides, the skin was wiped once with 70% ethanol. In the shaved area, linear incisions (1.5 cm in length) to the depth of the subcutaneous tissue were performed using sterile scalpels (Fig. 1A). Hemostasis was achieved by applying gentle pressure when necessary. The incision was sutured immediately using a 3.0 silk thread to approximate the cut edges of the skin (Fig. 1B) in one wound in each rat, meanwhile, the other wound was left without sutures.

Animals were closely observed for wound closure, and for the appearance of any signs of infection, and those displaying wound swelling or infection were separated, excluded from the study and replaced. The wounds and general health of the animals were monitored daily for up to 60 days post-incision.

After 1 through 60 days, rats were anaesthized by diethyl ether. The wounds with the surrounding tissues were excised after scarification of the rats by cervical dislocation 1, 2, 3, 4, 5, 7, 10, 14, 21, 28, 35, 48, 54 and 60 days after the skin incision.

2.3. The rats were divided into

A. *Antemortem inflicted skin wound ($n = 56$)*: As before four rats were sacrificed at each of the above mentioned 14 time points (i.e. $n = 4$ at each time point).
B. *Postmortem inflicted skin wound ($n = 6$)*: The rats in this group were sacrificed and postmortem wounds were inflicted within 30 min after death in the same region as the wounded groups after shaving the hair. The skin samples were collected 1 h and 3 h after the incision.
C. *Control group ($n = 4$)*: The control specimens were excised from uninjured rats in the same region as the wounded groups after shaving the hair.

2.4. Histopathological analysis

For histopathological study, the excised wound, together with the surrounding tissue was fixed in formalin and embedded in paraffin. Sections, 5um thick, were prepared and stained with hematoxylin and eosin (H&E). Sections of the excised wound tissue were also subjected to D2-40 immunohistochemistry as follows: sections were deparaffinized in xylene, rehydrated