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Local induction of lymphangiogenesis with engineered fibrin-binding VEGF-C promotes wound healing by increasing immune cell trafficking and matrix remodeling

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ABSTRACT

Lymphangiogenesis occurs in inflammation and wound healing, yet its functional roles in these processes are not fully understood. Consequently, clinically relevant strategies for therapeutic lymphangiogenesis remain underdeveloped, particularly using growth factors. To achieve controlled, local capillary lymphangiogenesis with protein engineering and determine its effects on fluid clearance, leukocyte trafficking, and wound healing, we developed a fibrin-binding variant of vascular endothelial growth factor C (FB-VEGF-C) that is slowly released upon demand from infiltrating cells. Using a novel wound healing model, we show that implanted fibrin containing FB-VEGF-C, but not free VEGF-C, could stimulate local lymphangiogenesis in a dose-dependent manner. Importantly, the effects of FB-VEGF-C were restricted to lymphatic capillaries, with no apparent changes to blood vessels and downstream collecting vessels. Leukocyte intravasation and trafficking to lymph nodes were increased in hyperplastic lymphatics, while fluid clearance was maintained at physiological levels. In diabetic wounds, FB-VEGF-C-induced lymphangiogenesis increased extracellular matrix deposition and granulation tissue thickening, indicators of improved wound healing. Together, these results indicate that FB-VEGF-C is a promising strategy for inducing lymphangiogenesis locally, and that such lymphangiogenesis can promote wound healing by enhancing leukocyte trafficking without affecting downstream lymphatic collecting vessels.

1. Introduction

Lymphatic vessels provide transport routes for immune cells, macromolecules and fluid from the periphery of the body to the lymph nodes and eventually back to the blood. In addition, lymphatic endothelial cells (LECs) can modulate immune cell trafficking and function by secreting chemokines, regulating surface and junctional adhesion molecules for leukocyte transmigration, expressing stimulatory and inhibitory receptors, and presenting antigens to T cells [1,2]. Lymphatic vessel expansion, also called lymphangiogenesis, is driven by multiple factors, including VEGF-C and VEGF-D, which are ligands of vascular endothelial growth factor receptor 3 (VEGFR-3) [3,4]. Lymphangiogenesis occurs post-developmentally in wound healing, chronic inflammation, and cancer [5].

In dermal wound healing, a tissue-deposited fibrin clot is initially infiltrated by innate immune cells (*i.e.*, neutrophils, monocytes, and macrophages) to be populated by anti-inflammatory macrophages and myofibroblasts. These healing-specific contractile cells cleave fibrin and remodel the new matrix in wounds, and their population declines when the mechanical tension is removed [6]. The appearance of myofibroblasts is followed by an

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Non-standard abbreviations and acronyms

FB-VEGF-C	Fibrin-binding variant of vascular endothelial growth factor C
LECs	Lymphatic endothelial cells
VEGFR-3	Vascular endothelial growth factor receptor 3
MMP	Matrix metalloproteinase
FXIIIa	Factor XIIIa
α_2-PI	α_2-plasmin inhibitor
GT	Granulation tissue
DC	Dendritic cells
BECs	Blood endothelial cells

ingrowth of transient blood vessels to meet increasing demands for oxygen and nutrients for the support and surveillance of tissue repair. Mechanisms of rapid restoration of blood circulation in wound healing include tension-dependent looping angiogenesis with sprouting and intussusception [7,8]. In contrast, lymphatic vessel regeneration is less well studied [9,10]. Therefore, even though it is well established that lymphangiogenesis follows angiogenesis in wound healing [11–13], the precise roles for the restored lymphatic vasculature in wound healing remain unclear.

We previously showed that increased interstitial fluid flow, such as that generated in inflamed or healing tissues, can stimulate myofibroblasts differentiation and matrix alignment [14]. In turn, this could increase directional wound contractility and vascularization, possibly through a force-dependent vessel looping mechanism that accelerates the healing process [6,15]. We assume that wound-associated lymphangiogenesis could alter immune cell trafficking from the wound, since lymphatic expression of adhesion molecules like E-selectin and ICAM-1 regulate leukocyte emigration [16], along with Lyve-1, a hyaluronan receptor expressed predominantly on initial lymphatics [17]. In turn, changes in leukocyte trafficking could affect the local profiles of cell-derived signals and the production of cytokines and growth factors that directly stimulate matrix remodeling.

Previously, it was shown that reduced lymphatic vessel formation contributes to impaired diabetic skin wound healing [18]. However, few studies have explored therapeutic lymphangiogenesis as a potential means to improve healing in such diabetic wounds, which are resistant to healing and develop high interstitial fluid pressure [19,20]. In models where lymphatic vessels were ablated, local delivery of recombinant VEGF-C was shown to improve healing and reduce tissue edema [21], and adenovirally delivered VEGF-C expedited reconnection of lymphatic vessels after lymph node dissection [19]. Interestingly, adenoviral overexpression of VEGF-C in skin diabetic wounds was shown to stimulate their healing, predominantly by the activation of blood vessel angiogenesis and local attraction of macrophages [20]. These results established the therapeutic potential of VEGF-C.

The local delivery of lymphangiogenic growth factors remains a challenge in clinical translation. To date, most studies with *in vivo* delivery of VEGF-C to induce lymphangiogenesis have used adenoviral vectors [10]. However, with viral vectors it is difficult to control dose, and the risk of unpredictable immune responses against the virus-based vehicles is an obstacle for patient translation [22]. For protein delivery, wild-type proteins rapidly diffuse from the injection site and cannot be maintained locally for more than a day [23], and repeated high-dose administration of VEGF-C could lead to edema and venous enlargement because higher doses of VEGF-C can induce pathological permeability and hyperplasia of blood vessels [24,25] as well as dysfunctional remodeling of collecting lymphatic vessels [19,26].

Over the last two decades, new approaches used in growth factor engineering for wound healing have been developed [23,27]. In particular, the immobilization of a growth factor to a biopolymer or to extracellular matrix components enables its local and cell-demanded release by augmenting its binding to cell surface receptors and creating local gradients that mimic those of endogenously released growth factors within the extracellular environment [28]. One well-established method is the recombinant fusion of a substrate sequence for the coagulation transglutaminase Factor XIIIa (FXIIIa), the substrate being derived from α_2-plasmin inhibitor (α_2-PI, where the substrate is α_2-$PI_{1\text{-}8}$), to exogenous growth factors. These growth factors can then be covalently attached to fibrin through FXIIIa during polymerization and are released only when fibrin is proteolytically cleaved during remodeling [29]. This approach diminishes the need for the application of high doses of growth factors and prevents growth factor toxicity. Furthermore, the fibrin matrix supports cell migration and proliferation and is completely cleared when healing is completed [30]. Nevertheless, no strategy that immobilizes VEGF-C within the fibrin matrix has been implemented for lymphangiogenic therapy.

Here, we developed a fibrin-binding VEGF-C variant, in which the FXIIIa substrate sequence α_2-$PI_{1\text{-}8}$ and a matrix metalloproteinase (MMP)-degradable domain are inserted, and explore its therapeutic applications. With this design, VEGF-C can be released by either the plasmin-mediated cleavage of fibrin or MMP-mediated cleavage of the substrate peptide fused between α_2-$PI_{1\text{-}8}$ and VEGF-C, referred to as FB-VEGF-C. We found that new functional initial lymphatic vessels could be specifically induced with a low-dose, single implantation of FB-VEGF-C in a subcutaneous cartilage-replacement healing model. The pro-lymphangiogenic effects were local and only affected lymphatic capillaries, not downstream collecting vessels, which further provided the opportunity to conduct a comprehensive study of the morphology and function of the newly formed lymphatic capillaries. To do that, we developed a functional assay that tests the ability of newly formed initial vessels to attract dendritic cells (DC) and further evacuate them from the healed wound to the draining lymph node. We also established an imaging method for the morphological analysis of the entire draining collecting vessel system, from the initial lymphatic bed down to the draining lymph node. Finally, we designed an assay that aimed to quantify lymphatic fluid drainage at the physiological interstitial fluid pressure, or without an injection swelling pressure, which would reveal dysfunctions in the lymphatic vasculature. Similarly to the newly formed lymphatics in control wounds, hyperplastic lymphatics in FB-VEGF-C-healed wounds attracted DCs that could transmigrate to the lymphatic lumen. The net migration towards the draining lymph node was increased via FB-VEGF-C-healed lymphatics, while the lymphatic clearance remained unchanged. Importantly, in diabetic mice, the local delivery of FB-VEGF-C in impaired wounds improved granulation tissue (GT) formation and increased the interaction of immune cells with activated lymphatic vessels. Together, these findings suggest that FB-VEGF-C is effective at inducing lymphangiogenesis locally, only within initial lymphatics, and such engineered local lymphangiogenesis holds therapeutic promise for impaired wound healing.

2. Methods

2.1. Mice

All experiments were carried out according to a protocol approved by the Committee for Animal Experiments for the Canton