REVIEW ARTICLE

Angiogenic and lymphangiogenic factors in wound healing

Authors
Kazuhiko Shimizu and Fumi Sato
Affiliations
Department of Anatomy, School of Medicine, Toho University, Tokyo, Japan; 5-21-16 Omori-Nishi, Ota-ku, Tokyo 143-8540, Japan.
Telephone Number: +81-3-3762-4151, Fax Number: +81-3-5493-5411
Corresponding Author:
Kazuhiko Shimizu
Department of Anatomy, School of Medicine, Toho University,
5-21-16 Omori-Nishi, Ota-ku, Tokyo 143-8540, Japan.

Email: kazuhiko.shimizu@med.toho-u.ac.jp

Telephone Number: +81-3-3762-4151, Fax Number: +81-3-5493-5411

ABSTRACT

When any tissue is damaged, multiple cells and tissues work towards the repair of the wounded site. Blood and lymphatic vessels are particularly important for the regeneration and healing of tissues. Angiogenesis is the process by which new blood vessels are formed. Angiogenesis is induced by angiogenic factors such as vascular endothelial growth factor (VEGF)-A which plays an important role in the repair of the damaged site. VEGF-A is expressed by macrophages, but pericytes also promote vascularization by expressing VEGF-A. In addition to VEGF-A, wound-related macrophages express tumor necrosis factor-α, Platelet-derived growth factor-bb, Interleukin (IL)-1, IL-6, and transforming growth factor β, and act on other cells. Pericytes change properties depending on the stage of the wound. For lymphangiogenesis, the expression of VEGF-C or -D, which are lymphatic endothelial growth factors, is the most important. Lymphatic vessel endothelial hyaluronan receptor-1-positive macrophages, which appear in the stroma, are also actively involved in lymphangiogenesis. On the other hand, podoplanin-positive myofibroblasts are indirectly involved in wound healing by being affecting in leukocyte migration as an “extravascular pathway”.

Keywords:: angiogenesis, lymphangiogenesis, wound healing, LYVE-1, podoplanin
dothelial dysfunction, ambulatory blood pressure, central blood pressure, prehypertension

Introduction

Wound healing is a complex process that combines multiple processes and has an inflammatory phase, proliferative phase, and remodeling phase. During the inflammatory phase, platelets aggregate and block the wound, and various cytokines and cell growth factors are secreted, causing several cells to infiltrate the wound. During the growth phase, epidermal cells, fibroblasts, and vascular endothelial cells proliferate, forming granulation tissue. Scars form during the remodeling phase. Blood and lymphatic vessels play a vital role in the wound healing processes. In this review document, we will discuss angiogenesis and lymphangiogenesis, and the various factors involved in wound healing.

1. Angiogenic factors and their expression

Angiogenesis is a complex system that is highly controlled in the body, and requires various factors to act in conjunction to be effective. Factors pertaining to angiogenesis in wound-sites include vascular endothelial growth factor (VEGF), angiopoietin (ANGPT), fibroblast growth factor (FGF), and transforming growth factor β (TGF-β). Co-expression of these growth factors is essential for angiogenesis. Keratinocytes, macrophages, fibroblasts, and pericytes (PCs) are among the cells that express the angiogenic factor. The interaction of these cells with vascular endothelial cells makes the process of wound healing intricate.

1.1 VEGF-A

VEGF has been isolated as a substance with two properties; it is a growth factor for vascular endothelial cells and also acts as a vascular permeability factor, known as VEGF-A [1-3]. VEGF-A has at least six known isoforms namely VEGF 121, 145, 165, 183, 189, and 206 [4,5]. VEGF-A is the most important angiogenic factor involved in wound healing. VEGF-A is a downstream protein of Hypoxia-inducible factor 1-alpha, and its expression is induced by hypoxia in wounds [6]. VEGF binds to vascular endothelial growth factor receptor (VEGFR)-1 (encoded by the FLT-1 gene) and/or VEGFR-2 (encoded by the Flk-1 gene), expressed in vascular endothelial cells, thereby promoting endothelial cell migration and proliferation. This induces angiogenesis and ensures the survival of the cells.

1.2 ANGPT

ANGPT belongs to the VEGF family and mainly regulates the adhesion of PCs to vascular endothelial cells. Both the agonist ANGPT-1, and the antagonist ANGPT-2, bind to the endothelial cell receptor, TIE2. ANGPT-1 is produced by PCs, and ANGPT-2 is produced by endothelial cells. ANGPT-1 attaches PCs to vascular endothelial cells to form a mature blood vessels, whereas ANGPT-2 releases PCs [7].

1.3 FGFs

Many types of FGFs have been reported (more than 23 homologs) of which FGF1 and FGF2 are particularly important for angiogenesis. FGF1 and FGF2 not only stimulate the proliferation of vascular endothelial cells, but also organize the vascular lumen structure [8]. Additionally, FGFs play an important role in the formation of granulation tissue in the process of wound healing.

1.4 TGF-β

TGF-β is a multifunctional cytokine with at least three isoforms (TGF-β1, -β2, and -β3). TGF-β controls cell growth, proliferation, differentiation, and apoptosis for various cell types. TGF-β also has many signaling pathways and promotes angiogenesis during wound healing [9].

1.5 Macrophages

Macrophages play an important role in the inflammatory phase of tissue repair [10]. Due

to their dynamic plasticity, macrophages can mediate both tissue destruction and repair [11]. Macrophages have many functions, defined by an intricate subset of cell-derived cytokines. Wound associated macrophages play an important role in angiogenesis. They not only express VEGF, but also promote VEGF production in keratinocytes and fibroblasts via tumor necrosis factor (TNF)-α, Platelet-derived growth factor (PDGF)-bb, Interleukin (IL)-1, IL-6, and TGF-β [12].

1.6 Keratinocytes

Increased amounts of VEGF in the wound area is as a result of migratory keratinocytes and macrophages in the granulation tissue [13]. Keratinocyte VEGF expression is indirectly promoted by macrophages expressing TNF-α and TGF-β [14].

1.7 Fibroblasts

Fibroblasts are stimulated by TGF-β to produce connective tissue growth factor (CTGF) [15]. CTGF induces connective tissue proliferation, vascular endothelial cell migration, and tube formation [16,17].

1.8 PCs

PCs, a type of wall cell in blood vessels, adhere to the outer circumference of capillaries and venules. Unlike vascular smooth muscle, PCs wrap around the basement membrane together with vascular endothelial cells and are in direct contact with endothelial cells. One of their functions is to stabilize blood vessels, but the mechanism by which this is achieved is still quite unclear [18]. It has been suggested that the function of PCs during angiogenesis is to suppress endothelial cell proliferation and to stabilize the vessel wall [19-21]. However, some studies have suggested that PCs can induce endothelial cell proliferation and sprouting during angiogenesis [22-30]. In addition, PCs exhibit heterogeneity at each stage of wound healing, and express VEGF-A to promote proliferation in the neovascular tip and surrounding regions of the endothelial cell [31]. The zebrafish model suggests that the wound activates PCs and induces angiogenesis [32].

2. Lymphangiogenic factors and their expression

Lymphatic vessels play an important role in tissue fluid collection and immunity transfer pathways. However, although the mechanism of lymphatic vessel formation and the *in vivo* regulatory factors affecting them have recently been studied, it still remains unclear.

The formation of lymphatic vessels during inflammation and wound healing, has also been reported and several influential factors identified. However, lymphatic vessels have not been studied as extensively as blood vessels. VEGF-C and -D are lymphatic endothelial growth factors, Prox-1 is a lymphatic endothelial cell master factor, and Foxc2 is important for lymphatic valve formation. Furthermore, lymphatic markers VEGFR3, LYVE-1, and podoplanin are also considered key. In particular, a large number of cells expressing lymphatic markers appear at the wound site, and the relationship between these cells and lymphangiogenesis may be important.

2.1 VEGF-C and -D

VEGF-C and -D, ligands for VEGFR3/Flt4, play an important role in the proliferation and migration of lymphatic endothelial cells [33]. VEGF-C and -D bind to VEGFR2 [34-36], and VEGFR2 and VEGFR3 can also form heterodimers [37,38]. In wound healing, coordinated signaling of VEGFR2 and VEGFR3 is thus a key factor [39].

2.2 Prospero homeobox protein 1 (PROX1)

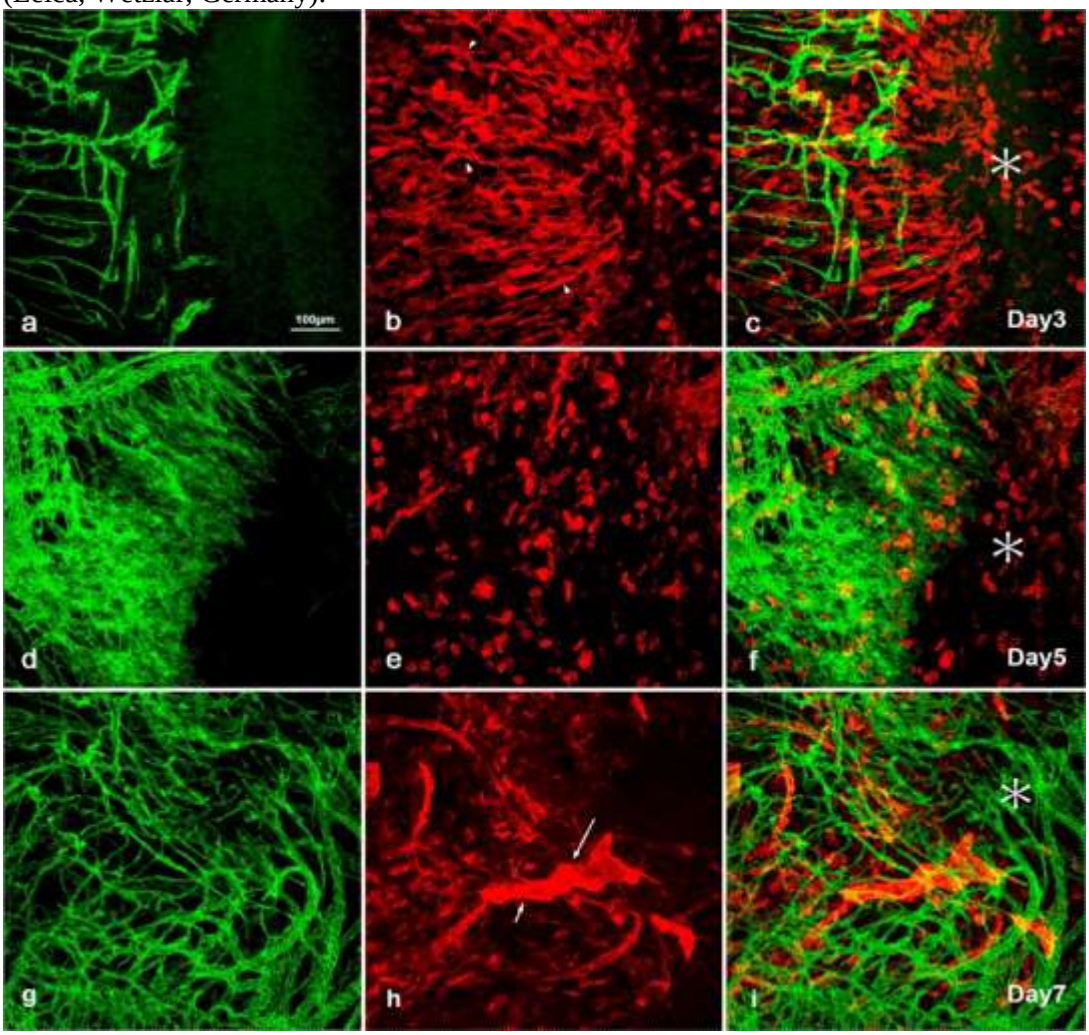
PROX1 is an essential master transcription factor for the development and maintenance of lymphatic vessels [40-42]. Binding of VEGFC, D, and VEGFR3 activates PROX1. In a corneal injury model, PROX1 was suppressed by microRNA miR-466, suggesting that lymphangiogenesis could be suppressed [43]. The functionality of PROX1

in wound healing is expected to develop in the future.

2.3 Forkhead box protein (FOX)C2

FOXC2, is highly expressed in the fetus and adult lymphatic vessels [44,45]. In the fetus, FOXC2 is involved in the formation of lymphatic vessels in coordination with FOXC1 [46]. In mature lymphatic vessels, FOXC2 is downregulated, resulting in decreased expression levels of PROX1, VEGFR-3, and LYVE-1 [47]. However, the expression and function of FOXC2 during lymphangiogenesis in wounds is still unknown.

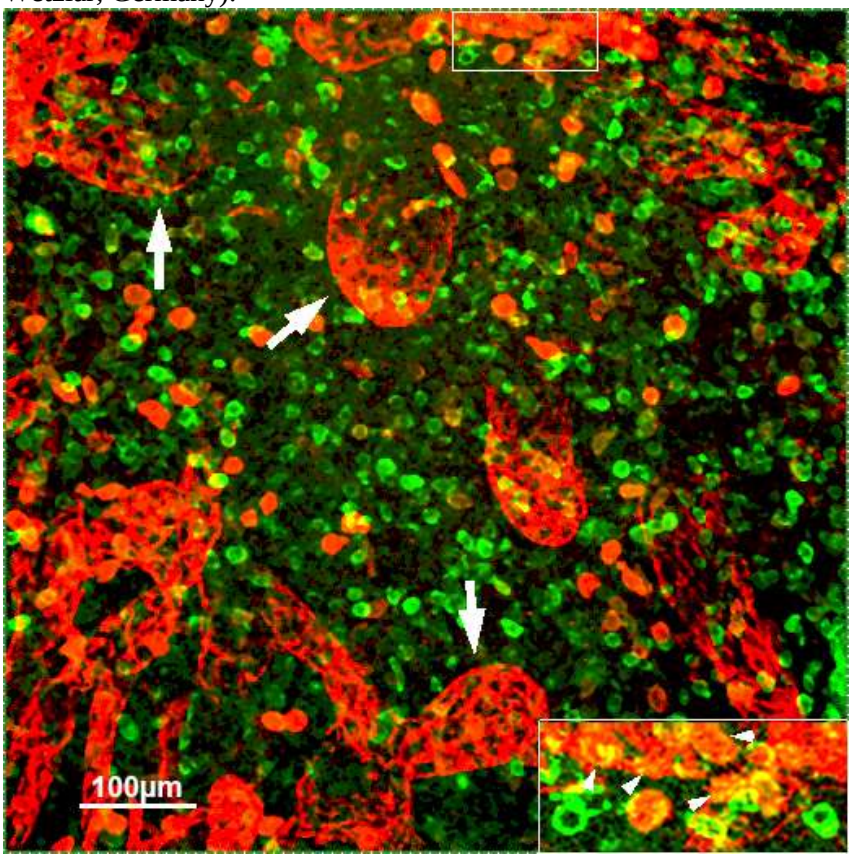
Figure 1: Double immunofluorostaining in the wound section of the skin in a mouse-model (C57BL / 6N, 8-week-old male).
a-c: third day after injury, d-f: fifth day after injury, g-i: seventh day after injury. Green: CD31, red : LYVE-1, *: wound area, scale bar = 100μm.
On the third day after injury, many LYVE-1-positive cells were seen to appear. LYVE-1 positive cells (represented by the arrow markings) extended in row towards the center of the wound (a-c).
On the fifth day after injury, abundant new blood vessels were seen (d), but LYVE-1-positive cells did not line up as they had done on the third day.
Seven days after injury, blood vessels formed a network (g), and lymphatic-like structures could be observed (h, represented by arrows).
These fluorescent images were observed under a Leica TCS-SL confocal laser scanning microscope (Leica, Wetzlar, Germany).



2.4 LYVE-1 / LYVE-1^{+} cells

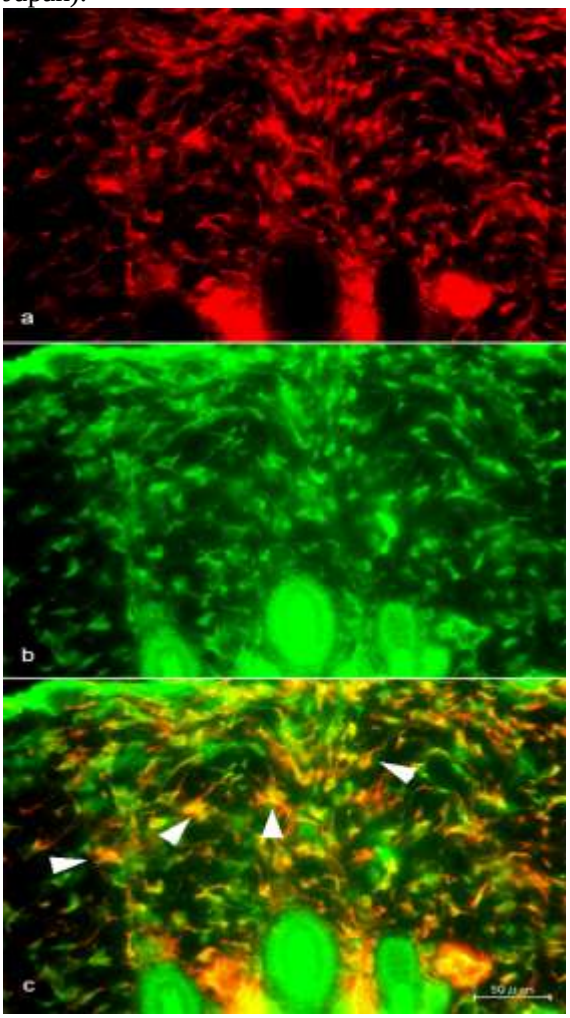
LYVE-1, a lymphatic endothelial cell marker, is a hyaluronic acid receptor and a CD44 homolog [48]. Moreover, LYVE-1 is expressed in sinusoids, macrophages, and several cells [49]. In order to observe lymphangiogenesis during wound healing, a full-thickness defect was made in the skin of mice. It was stained and observed for LYVE-1, CD31, and CD11b (Figure 1 and 2). On the third day after injury, LYVE-1 positive macrophages accumulated to form a lymphatic-like structure (Figure 1 a-c), but on the fifth day, this structure deteriorated (Figure 1 d-f). On the 7th day after injury, normal lymphatic vessels were observed (Figure 1g-i).

Figure 2: Double immunofluorescent staining in the wound section of the skin in a mouse-model (C57BL / 6N, 10-week-old male) on the 17th day after injury. Green: CD11b, red: LYVE-1, scale = 100μm. The blind ends of the lymphatic vessels were confirmed at the site of the wound on the 17th day after the injury (marked by arrows). In addition, CD11b-positive cells were seen to form the lymphatic endothelium (arrow markings in the figure inset; enlarged and highlighted by a white rectangle). These fluorescent images were observed under a Leica TCS-SL confocal laser scanning microscope (Leica, Wetzlar, Germany).



We also observed that some CD11b-positive macrophages were integrated into the lymphatic endothelium (Figure 2). On the 17th day, many blind ends of lymphatic vessels were observed, and the number of LYVE-1-positive macrophages was also seen to have decreased. Maruyama et al. had previously reported that macrophages had transdifferentiated and been incorporated into the lymphatic endothelial cells in the cornea of mice [50]. Similarly, in the skin, macrophages may have differentiated to form a part of the lymphatic endothelial cells.

Figure 3: Double immunofluorescent staining in the wound section of the skin in a mouse-model (C57BL / 6N, 8-week-old male) on day 1 after injury. Green: α-SMA, red: PDPN, scale = 50μm. Many PDPN positive cells appeared on the first day after injury (a). These cells had many cell processes. In addition, almost all PDPN positive cells co-expressed α-SMA (b, c; represented by arrows). These fluorescent images were observed under a KEYENCE BZ-9000 HS all-in-one microscope (KEYENCE, Osaka, Japan).



2.5 Podoplanin (PDPN) / PDPN$^+$ cells

PDPN is among the most commonly used lymphatic markers along with LYVE-1 and VEGFR3. PDPN expression in lymphatic endothelial cells is regulated by PROX1. In the development of lymphatic vessels, PDPN has proven to be essential for isolating lymphatic budding from veins [51,52]. In wound healing, PDPN is expressed in epithelial basal cells and is involved in epidermal cell migration [53]. We also found that many PDPN$^+$ cells appeared in the stroma using a mouse model to study wound healing. These cells were αSMA$^+$ myofibroblasts (Figure 3). Myofibroblasts differentiate from fibroblasts and epidermal cells. However, the origin of PDPN$^+$ myofibroblasts is unknown. PDPN$^+$ fibroblasts are involved in leukocyte migration as an "extravascular pathway" in lymphoid organs [54,55]. In light of these, PDPN$^+$ cells may not be directly involved in lymphangiogenesis, but may be indirectly involved in overall wound healing by causing the migration of various cells.

3. Summary

Angiogenesis and lymphangiogenesis are important processes in wound healing. Angiogenesis has been the focus of a lot of research; many manuscripts have studied embryology, tumors, and wound healing in various fields of study such as the molecular biology, physiology, pharmacology, and microanatomy. However, lymphangiogenesis is yet to be explored. Additionally, the contribution of cells which act as lymphatic markers involved in wound healing could be an interesting aspect for further research.

4. Acknowledgments

The authors would like to thank Ms. Ayaka Shibano, Dr. Sachiko Miyamoto-Kikuta, Dr. Masae Morishima, Dr. Shuji Kitahara and Professor Taichi Ezaki for their advice and contribution to this report. This work was supported by Grant-in-aid for Scientific Research (C) (JSPS KAKENHI Grant numbers JP15K10953 and JP18K09496).

5. Conflicts of interest

The authors declare no conflicts of interest associated with this manuscript.

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