

# Role of angiogenesis and angiogenic factors in acute and chronic wound healing

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## ABSTRACT

Angiogenesis plays a crucial role in wound healing by forming new blood vessels from preexisting vessels by invading the wound clot and organizing into a microvascular network throughout the granulation tissue. This dynamic process is highly regulated by signals from both serum and the surrounding extracellular matrix environment. Vascular endothelial growth factor, angiopoietin, fibroblast growth factor and transforming growth factor-beta are among the potent angiogenic cytokines in wound angiogenesis. Specific endothelial cell ECM receptors are critical for morphogenetic changes in blood vessels during wound repair. In particular integrin ( $\alpha v \beta 3$ ) receptors for fibrin and fibronectin, appear to be required for wound angiogenesis:  $\alpha v \beta 3$  is focally expressed at the tips of angiogenic capillary sprouts invading the wound clot, and any functional inhibitors of  $\alpha v \beta 3$  such as monoclonal antibodies, cyclic RGD peptide antagonists, and peptidomimetics rapidly inhibit granulation tissue formation. In spite of clear knowledge about influence of many angiogenic factors on wound healing, little progress has been made in defining the source of these factors, the regulatory events involved in wound angiogenesis and in the clinical use of angiogenic stimulants to promote repair.

## Key words:

Angiogenic factors, endothelium, extracellular matrix protein, granulation tissue, wound healing

## INTRODUCTION

Neovascularization or angiogenesis is important for wound healing as it involves the growth of new capillaries to form granulation tissue.<sup>[1-4]</sup> Three to five days after tissue injury, new capillaries become visible in the wound bed as granulation tissue, which acts as a matrix for proliferating blood vessels, migrating fibroblasts and new collagen.<sup>[5]</sup> Impaired granulation is a hallmark of chronic wounds encountered with diabetes and venous or arterial insufficiency.

In 1960s, research began in the field of angiogenesis to determine how new blood vessels enhance solid tumor growth.<sup>[6]</sup> Physiologists later discovered that neovascularization occurs during tissue regeneration.<sup>[7]</sup> Proliferating capillaries bring oxygen and micronutrients to growing tissues and remove catabolic waste products. These vessels are present in the endothelium that secretes paracrine factors to promote survival of adjacent cells by preventing apoptosis or programmed cell death.<sup>[8]</sup> Because

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angiogenesis is required for wound healing, its induction is beneficial in many clinical situations for achieving wound closure.

## PHYSIOLOGICAL CONTROL OF ANGIOGENESIS

Angiogenesis plays a critical role in wound healing. By developing capillary sprouts, which digest endothelial cells and invade the extracellular matrix (ECM) stroma after penetrating through the underlying vascular basement membrane (VBM), and form tube-like structures that continue to extend, branch, and form networks. During angiogenesis capillary advancement in ECM occurs by endothelial cell proliferation and direction of growth is guided by chemotaxis from the target region. The interaction among endothelial cells, angiogenesis factors and surrounding ECM proteins is temporally and spatially synchronized.<sup>[9,10]</sup>

Angiogenesis can be induced in response to injury via pro- and anti-angiogenic factors present throughout the body. Pro-angiogenic factors consist of thrombin, fibrinogen fragments, thymosin- $\beta$ 4 and growth factors. Angiogenic growth factors are stored in platelets and inflammatory cells that circulate in the bloodstream, and are sequestered within the ECM. The production of these factors is regulated by genes expressed in response to hypoxia and inflammation, such as hypoxia-inducible factors (HIF) and cyclooxygenase-2 (COX-2).<sup>[11-13]</sup> In contrast, angiogenesis inhibitor factors suppress blood vessel growth.<sup>[14,15]</sup> Some inhibitors circulate in the blood stream at low physiological levels while others are stored in the ECM surrounding blood vessels. Vascular growth is suppressed when there is a physiological balance between angiogenesis stimulators and inhibitors.<sup>[15]</sup> Immediately following injury, however, angiogenic stimuli are released into the wound bed, and a shift occurs in regulators favoring vascular growth [Figure 1].

## THE ANGIOGENESIS CASCADE

Angiogenesis occurs as an orderly cascade of molecular and cellular events in the wound bed:

1. Endothelial cell surface has receptors to which angiogenic growth factors bind in preexisting venules (parent vessels);
2. Growth factor-receptor binding activates signaling pathways within endothelial cells;

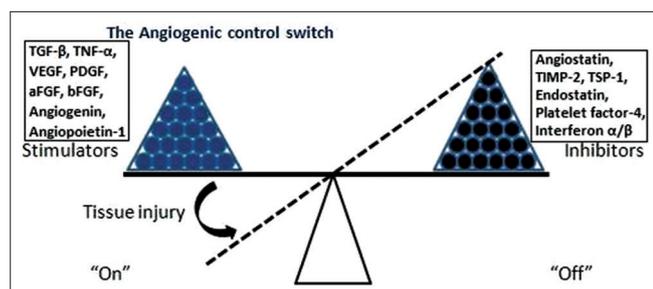


Figure 1: Angiogenesis is a balance between stimulators (growth factors) and inhibitors as shown in this model

3. Proteolytic enzymes released by activated endothelial cells dissolve the basement membrane of surrounding parent vessels;
4. Endothelial cells proliferate and sprout outward through the basement membrane;
5. Endothelial cells migrate into the wound bed using integrins ( $\alpha$ v $\beta$ 3,  $\alpha$ v $\beta$ 5 and  $\alpha$ v $\beta$ 1) which are cell surface adhesion molecules;
6. Matrix metalloproteinases (MMPs) dissolve the surrounding tissue matrix in the path of sprouting vessels;
7. Vascular sprouts form tubular channels that connect to form vascular loops;
8. Vascular loops differentiate into afferent (arterial) and efferent (venous) limbs;
9. New blood vessels mature by recruiting mural cells (smooth muscle cells and pericytes) to stabilize the vascular architecture;
10. Blood flow begins in the mature stable vessel.

These complex growth factor-receptor, cell-cell and cell-matrix interactions characterize the angiogenesis process, regardless of the stimuli or its location in the body.

## THE ANGIOGENESIS MODEL OF WOUND HEALING

Wound healing occurs in four major overlapping stages: (1) hemostatic, (2) inflammatory stage, (3) proliferative stage, and (4) remodeling stage. Although granulation is assigned to the proliferative stage, angiogenesis is initiated immediately after tissue injury and is mediated throughout the wound healing process.

### Step 1: Angiogenesis initiation

Basic fibroblast growth factor (bFGF) stored within intact cells and the ECM is released from damaged tissue.<sup>[16]</sup> Bleeding and hemostasis in a wound also initiate angiogenesis. Cellular receptors for vascular endothelial growth factor (VEGF) are upregulated by thrombin in the wound.<sup>[17]</sup> Endothelial cells exposed to thrombin also release gelatinase A (MMP-2), which promotes the local dissolution of basement membrane, a necessary early step of angiogenesis.<sup>[18]</sup> Platelets release multiple growth factors, including platelet-derived growth factor (PDGF), VEGF, transforming growth factor (TGF- $\alpha$ , TGF- $\beta$ ), bFGF, platelet-derived endothelial cell growth factor and angiopoietin-1 (Ang-1). These factors stimulate endothelial proliferation, migration and tube formation.<sup>[19-22]</sup>

### Step 2: Angiogenesis amplification

Macrophages and monocytes release numerous angiogenic factors, including PDGF, VEGF, Ang-1, TGF- $\alpha$ , bFGF, interleukin-8 (IL-8) and tumor necrosis factor alpha into the wound bed during the inflammatory phase amplifying angiogenesis further.<sup>[23,24]</sup> Several growth factors (PDGF, VEGF and bFGF) synergize in their ability to vascularize tissues.<sup>[25]</sup> Proteases that break down damaged tissue matrix further release matrix-bound angiogenic stimulators. Enzymatic cleavage of fibrin yields fibrin fragment E, which stimulates angiogenesis directly and also enhances the

effects of VEGF and bFGF.<sup>[26]</sup> Expression of the inducible COX-2 enzyme during the inflammatory stage of healing also leads to VEGF production and other promoters of angiogenesis.<sup>[27]</sup>

### Step 3: Vascular proliferation

Hypoxia is an important driving force for wound angiogenesis. Expression of gene HIF-1 $\alpha$ , due to hypoxic gradient between injured and healthy tissue triggers VEGF production.<sup>[24,28]</sup> VEGF is present in both wound tissue and exudate.<sup>[28,29]</sup> VEGF is also known as vascular permeability factor since it increases permeability of capillaries.<sup>[30]</sup> Hypoxia also leads to endothelial cell production of nitric oxide (NO). NO promotes vasodilation and angiogenesis to improve local blood flow.<sup>[31]</sup>

### Step 4: Vascular stabilization

Vascular stabilization is governed by Ang-1, tyrosine kinase with immunoglobulin-like and EGF-like domains 2 (Tie-2), smooth muscle cells and pericytes. Production of PDGF and recruitment of smooth muscle cells and pericytes to the newly forming vasculature are regulated by binding of Ang-1 to its receptor Tie-2 on activated endothelial cells.<sup>[32-34]</sup> A PDGF deficiency leads to poorly-formed immature blood vessels.<sup>[35]</sup>

### Step 5: Angiogenesis suppression

Angiogenesis is suppressed at the terminal stages of healing.<sup>[36]</sup> As tissue hypoxia is restored, and inflammation subsides, the level of growth factors decline in the wound. Pericytes which stabilize endothelial cells secrete an inhibitory form of activated TGF- $\beta$  that impedes vascular proliferation.<sup>[34,37,38]</sup> A cleavage product of collagen XVIII, endostatin, is present surrounding the VBM, and it inhibits wound vascularity.<sup>[39,40]</sup>

## WOUND ANGIOGENIC STIMULATORS AND INHIBITORS

A number of angiogenic stimulators have been identified in wound and others are likely to exist that play an important role in the repair [Table 1]. The stimulators in wound fluids are growth factors known to increase endothelial cell migration and proliferation *in vitro*.<sup>[41]</sup>

The FGF comprises of 23 homologous structures that are small polypeptides with a central core containing

140 amino acids. Acidic FGF and bFGF are the first few to be discovered and are now designated as FGF-1 and FGF-2, respectively.<sup>[42]</sup> Both are preferentially involved in the process of angiogenesis.<sup>[43,44]</sup> These compounds are polypeptides of about 18 kDa, single chained and nonglycosylated. They transmit their signals through FGF receptor-4 (FGFR-4) high-affinity, protein family of transmembrane tyrosine kinases (FGFR-1 to FGFR-4), that bind to different FGFs with different affinities. The strong interactions of FGF-1 and FGF-2 with glycosaminoglycans, such as heparin sulfate present in the ECM,<sup>[45]</sup> makes the FGFs stable against thermal, proteolytic denaturation and limits its diffusibility. Thus, the ECM acts as a reservoir for pro-angiogenic factors. Most members of the FGF family act as a broad spectrum mitogen that stimulates the proliferation of mesenchymal cells of mesodermal origin, as well as ectodermal and endodermal cells.

FGF-1 and FGF-2 are synthesized by a variety of cell types including inflammatory cells and dermal fibroblasts that are involved in angiogenesis and wound healing. When liberated from ECM, they act on the endothelial cells in a paracrine manner, or when released by endothelial cell they act in an autocrine manner promoting cell proliferation and differentiation. During the formation of granulation tissue, FGF-2 promotes cell migration through surface receptors for integrins, which mediate the binding of endothelial cells to ECM.<sup>[44]</sup>

Vascular endothelial growth factor increase vaso-permeability by increasing the fenestration and hydraulic conductivity. This allows leakage of fibrinogen and fibronectin, which are essential for the formation of the provisional ECM.<sup>[46-48]</sup> The ECM is produced in large quantities by the epidermis during wound healing.<sup>[49]</sup> Low oxygen tension that occurs in tissue hypoxia is a major inducer of VEGF<sup>[50]</sup> and its receptors.<sup>[51]</sup> Thus, cell disruption and hypoxia appear to be strong initial inducers of potent angiogenesis factors at the wound site. VEGF family currently includes VEGF-A, VEGF-B, VEGF-C, VEGF-D, VEGF-E and placental growth factor.<sup>[52]</sup> VEGF-A is a homodimer glycoprotein whose subunits are linked by 2 disulfide bonds. VEGF-A is synthesized from internal rearrangements (“alternative splicing”) of mRNA. Thus, there is the production of 7 isoforms with 121 to 206 amino acids.<sup>[53-55]</sup> Among these, the VEGF121, VEGF165, VEGF189 and VEGF206 are the predominant isoforms.<sup>[56]</sup> These isoforms show similar biological activities, but differ in their binding properties to heparin and ECM.<sup>[57]</sup>

Vascular endothelial growth factor is a potent vascular endothelial cell-specific mitogen that stimulates endothelial cell proliferation, microvascular permeability and regulates of several endothelial integrin receptors during sprouting of new blood vessels.<sup>[58]</sup> Furthermore, VEGF also acts as a survival factor for endothelial cells by inducing the expression of an anti-apoptotic protein B-cell lymphoma 2.<sup>[59]</sup>

TGF- $\beta$  stimulates the formation of granulation tissue by acting as a chemoattractant for neutrophils, macrophages and fibroblasts. Hence, TGF- $\beta$  is an important modulator of angiogenesis during wound healing by regulating cell

**Table 1: Angiogenic stimulators and inhibitors**

Stimulators	Inhibitors
aFGF (FGF-1)	Thrombospondin-1
bFGF (FGF-2)	Tissue inhibitors of matrix metalloproteinases
TGF- $\alpha$	Interferon alpha/beta/gamma
TGF- $\beta$	Angiostatin
PGE2	Endostatin
TNF- $\alpha$	
VEGF	
EGF	

FGF: Fibroblast growth factor, aFGF: Acidic fibroblast growth factor, bFGF: Basic fibroblast growth factor, TGF- $\alpha$ : Transforming growth factor-alpha, TGF- $\beta$ : Transforming growth factor-beta, VEGF: Vascular endothelial growth factor, EGF: Endothelial growth factor, PGE2: Prostaglandin E2

proliferation, migration, capillary tube formation and deposition of ECM.<sup>[60,61]</sup>

The angiopoietins are members of the VEGF family, which is largely specific for vascular endothelium. They include a naturally occurring agonist, Ang-1, and antagonist, Ang-2, both of which act by means of the Tie-2 receptor. Two new angiopoietins, Ang-3 in mice and Ang-4 in humans, have been identified, but their function in angiogenesis is unknown.<sup>[62]</sup>

Mast cell tryptase, stored in granules of activated mast cells, is an additional angiogenesis factor that directly degrades the ECM components or release matrix-bound growth factors by its proteolytic activity,<sup>[63,64]</sup> and acts indirectly by activating latent matrix metalloproteases. The addition of tryptase to microvascular endothelial cells cultured on a basement membrane matrix (matrigel) caused a marked increase in capillary growth. Furthermore, tryptase can induce endothelial cell proliferation in a dose-dependent manner, whereas specific tryptase inhibitors suppress the capillary growth.<sup>[65]</sup>

## IMPAIRED ANGIOGENESIS IN CHRONIC WOUNDS

Angiogenesis is impaired in all chronic wounds leading to further tissue damage results from chronic hypoxia and impaired micronutrient delivery. Specific defects have been identified in diabetic ulcers, venous insufficiency ulcers and ischemic ulcers.

### Diabetic ulcers

Patients with diabetes show abnormal angiogenesis in various organs. Vasculopathies associated with diabetes include abnormal blood vessel formation (e.g. retinopathy, nephropathy) and accelerated atherosclerosis leading to coronary artery disease, peripheral vascular disease, and cerebrovascular disease.<sup>[65]</sup> However, in diabetics, angiogenesis is decreased<sup>[66]</sup> resulting in poor formation of new blood vessels and thus decreased entry of inflammatory cells and their growth factors. Growth factors such as FGF-2 and PDGF, essential for wound healing have been found to be reduced in experimental diabetic wounds models.<sup>[67-70]</sup> Furthermore, in rat models, topical administration of high glucose to wounds was shown to inhibit the normal angiogenic process,<sup>[71]</sup> suggesting a direct role for high glucose levels in diminished angiogenesis.

Vascular endothelial growth factor plays an important role in vascular growth and has been shown to be deficient in diabetic wounds in experimental and clinical models.<sup>[72]</sup> Studies have shown that modulation of oxidative damage<sup>[73]</sup> or inhibition of the receptors for advanced glycation end products<sup>[74]</sup> improve wound healing and were associated with the up-regulation of endogenous VEGF. Moreover, VEGF administration improves wound healing in nondiabetic ischemic wounds<sup>[75]</sup> and blocking VEGF with neutralizing antibodies impedes tissue repair.<sup>[76]</sup> These studies support the notion that VEGF is critical for repair in impaired healing states and that the addition of VEGF could have a potential clinical use.<sup>[77]</sup> In fact,

Galiano *et al.*<sup>[78]</sup> found that topical VEGF accelerates wound healing in a diabetic mouse model.

Weinheimer-Haus *et al.*<sup>[79]</sup> found that low intensity vibration (LIV) applied vertically at 45 Hz with peak acceleration of 0.4 g for 30 min a day for 5 days a week starting on the day of injury in diabetic mice increases expression of pro-healing growth factors and chemokines (insulin-like growth factor-1, VEGF and monocyte chemoattractant protein-1) in wound environment. Though there was no evidence of a change in the phenotype of CD11b+ macrophages, however, LIV resulted in trend toward a less inflammatory phenotype in the CD11b2 cells which comprised of fibroblasts, endothelial cells and/or keratinocytes. These findings indicate that LIV may exert beneficial effects on wound healing by enhancing angiogenesis and granulation tissue formation, and these changes are associated with an increase in pro-angiogenic growth factors.<sup>[79]</sup>

### Venous insufficiency ulcers

Venous insufficiency ulcers or venous stasis ulcers result from incompetent valves in lower extremity veins, leading to venous stasis and hypertension that makes the skin susceptible to ulceration. Pathological findings associated with venous stasis ulcers include microangiopathy, fibrin “cuffing” and trapping of leukocytes within the microvasculature.<sup>[80,81]</sup>

Chronic venous stasis ulcer patients have elevated levels of VEGF in their circulation.<sup>[82]</sup> This may explain the vascular permeability and increased transudation of serum fluid in their wounds. Biopsies of these ulcers reveal microvessels that are surrounded by fibrin cuffs composed of fibrin and plasma proteins, such as  $\alpha$ -macroglobulin, thought to compromise gas exchange.<sup>[83-85]</sup> Clinical studies have shown that transcutaneous oxygen tension may be up to 85% lower in venous stasis ulcers compared with normal skin regions.<sup>[86]</sup> VEGF expression is up-regulated by hypoxia, which further exacerbates vascular permeability, formation of pericapillary fibrin cuffs and compromised gas exchange, which ultimately reduces growth factor availability in the wound.<sup>[87,88]</sup> VEGF promotes the formation tortuous, aberrant glomeruloid-like vascular structures found in granulation tissue.<sup>[89]</sup> Laboratory animals treated with VEGF form these glomeruloid vascular structures within 3 days and are characterized by poor perfusion.<sup>[90]</sup> In venous ulcers, the persistence of glomeruloid vessels may interfere with oxygen delivery and delay healing. In chronic venous stasis ulcers, high levels of proteases such as neutrophil elastase, MMPs and urokinase-type plasminogen activator are present.<sup>[91]</sup> Concomitantly, there are decreased levels of protease inhibitors, such as plasminogen activator inhibitor-2. Excessive protease activity may degrade the growth factors and destroy granulation tissue.

### Ischemic ulcers

Peripheral arterial disease (PAD) may result in severe ischemia.<sup>[92]</sup> Reduce tissue perfusion due to ischemia results in progressive tissue hypoxia, ischemia, necrosis and skin breakdown. In theory, tissue hypoxia should

initiate angiogenesis via inducing an HIF-1 $\alpha$  and angiogenic growth factors. In patients with PAD, serum levels of hepatocyte growth factor are elevated than in normal subjects.<sup>[93]</sup> The tissue compromise caused by severe macrovascular disease, however, may over dominate the angiogenic response. Inter-individual differences in the ability to mount angiogenesis under hypoxic conditions also exist among patients with atherosclerosis. Such variations may explain that patients with PAD are unable to generate adequate collateral circulation and unable to heal arterial ulcers despite surgical bypass. Therapeutic growth factors or other methods designed to stimulate angiogenesis might benefit patients with a defective angiogenic capacity. VEGF gene transfer<sup>[94]</sup> or autologous transplantation of bone marrow-derived endothelial progenitor stem cells<sup>[95]</sup> improved healing of arterial ulcers in patients.

## ANGIOMODULATORY STRATEGIES

Wound angiogenesis represents a realistic model to study molecular mechanisms involved in the formation and remodeling of vascular structures. In particular, the repair of skin defect offers an ideal model to analyze angiogenesis as it is easy to control and manipulate this process.<sup>[96]</sup> Vessel growth is controlled by the local actions of chemical mediators, the ECM, metabolic gradients and physical forces. Manipulation of some of these factors is being tried to improve healing in experimental wounds.<sup>[97]</sup> Scientists are working on mathematical models which describe the role of angiogenesis as observed during (soft tissue) wound healing. Through this model manipulation of the capillary tip, macrophage-derived chemical attractant profile, extracellular matrix and fibroblast diffusion coefficient may be analyzed to enhance wound healing.<sup>[98]</sup>

## CONCLUSION

Angiogenesis is a physiological process that is vital for normal wound healing. A number of factors regulate wound angiogenesis, including hypoxia, inflammation and growth factors. The molecular and cellular events in angiogenesis have been elucidated, and defects in this process are present in chronic wounds. Based on this knowledge, new wound healing strategies are emerging to deliver growth factors to the wound bed. Surgeons and other wound-care specialists can use this knowledge to identify defects and select interventions that may promote improved wound granulation and healing.

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