Inflammatory and Angiogenic Abnormalities in Diabetic Wound Healing: Role of Neuropeptides and Therapeutic Perspectives

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Abstract: Diabetic foot ulceration (DFU) is one of the most costly and debilitating complications of diabetes and is the leading cause of non-traumatic amputations, affecting 15% of the diabetic population. Impaired wound healing in diabetic patients without large-vessel disease has been attributed to microvascular dysfunction, neuropathy, and abnormal cellular and inflammatory responses. These abnormalities have been examined mainly in animal models although a few studies have been undertaken in diabetic patients. This review provides an overview of the inflammatory and vascular abnormalities in DFU and emphasises the role of angiogenic growth factors, endothelial progenitor cells (EPCs), and neuropeptides as mediators of wound healing and potential therapeutic agents for these chronic, non-healing ulcers.

Keywords: Wound healing, inflammation, angiogenesis, neuropeptides.

INTRODUCTION

Diabetes *mellitus* (DM) is a serious problem of public health worldwide and it tends to increase in numbers, mainly in the developed countries. In the USA, recent data refer that 8% of the general population and 25% of the population over the age of 65 is diabetic [1]. One of the most serious and debilitating complications of diabetes is the development of chronic non-healing foot ulcerations. Diabetic foot ulceration (DFU) is estimated to occur in 15% of diabetic patients, often requires prolonged hospitalizations for its management and is the major cause of disease-associated amputations in the western world [1-3].

Wound healing is an innate host response for restoration of tissue integrity. It is a complex process encompassing a number of coordinated steps, including homeostasis/coagulation, inflammation, migration-proliferation and remodeling [4]. After a skin break is produced, leakage of blood from blood vessels occurs and a fibrin clot is formed, plugging the defect. This provides an immediate, provisional repair and initiates a cascade of events that culminate to wound closure. In this process, the aggregated platelets release cytokines and growth factors that recruit neutrophils and monocytes. Fibroblasts, epithelial cells and endothelial cells (EC) also migrate to the wound site to form a contractile granulation tissue that brings the wound margins closer. Meanwhile, a fresh surface epithelium covers the wound surface and the granulation tissue differentiates progressively approaching the structural and functional characteristics of the mature dermis, finally repairing the lost tissue. This cascade implies the orderly initiation and arrest of many complex biological phenomena including cell proliferation, cell migration, cell differentiation and extracellular matrix (ECM) deposition [5]. The coordinated actions of cell and matrix signals orchestrate these processes.

The pathophysiologic relationship between diabetes and impaired healing is complex. In contrast to acute wounds that progress through the phases of wound healing linearly in healthy individuals, chronic wounds in diabetic patients become stalled in different phases and progression does not occur in synchrony due to diabetes associated neuropathy, microangiopathy and impaired immune function [4,6].

Studies in transgenic animal models null for specific genes coding for molecules known to play a role in wound healing, as well as wound healing research in embryos and the emerging cell-based strategies, have shown to be useful in the understanding of wound healing mechanisms.

However, further investigation is needed in order to: i) identify the precise molecular signals that regulate cell activity in a particular phase, ii) modulate cells/matrix molecules to stimulate wound healing, and iii) clarify the mechanisms of wound healing failure in chronic skin wounds.

This review focuses on the inflammatory and vascular abnormalities in DFU and emphasises the role of neuropeptides, angiogenic growth factors and EPCs as potential therapeutic agents for these chronic, non-healing ulcers.

CAUSES OF DIABETIC FOOT ULCERATION

Both acute and long-term occurrence of type 1 and type 2 Diabetes mellitus can result in complications of the neuronal and vascular systems. Since DM affects small and large blood vessel, it is known to cause various micro and macrovascular complications. The incidence of microvascular complications, specifically, nephropathy, retinopathy and peripheral neuropathy increases with the duration of diabetes. Diabetes-associated macrovascular diseases, namely, coronary artery disease, cerebrovascular disease and periph-

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eral vascular disease occur mainly as a result of accelerated atherosclerosis. Peripheral vascular disease (PVD) and peripheral neuropathy (PN) are the leading causes of DFU.

Peripheral vascular disease is a broad term that includes any disorder of the peripheral circulatory system, although it is often used as a synonym for peripheral arterial disease (PAD). According to different studies, the prevalence of PAD in patients with DFU varies from 10% to 60% [7-10]. In fact, a recent Eurodiale study, which was conducted in 14 European centers, has shown that the presence of PAD is a strong predictor of chronic, nonhealing foot ulcers [11]. In a previous study, Jude and colleagues [12] reported that the severity of PAD can be associated with higher rates of lower extremity amputation, morbidity and mortality.

Peripheral neuropathy is characterized by a progressive loss of nerve fibers that will eventually lead to loss of pain perception. It is now well established that PN is a key factor in the development of DFU. Also, PN is reported in 30 to 50% of diabetic patients. The risk factors for diabetic neuropathy include hyperglycemia, hyperlipidemia, high blood pressure, obesity, age over 40, and long-term occurrence of DM [13,14].

It is estimated that peripheral neuropathy increases the risk of DFU by 8- to 18- fold and the risk of lower extremity amputation by 2- to 15- fold. The mechanisms through which PN triggers the pathogenic pathway for ulceration are complex. The reduction of protective sensitivity, including sensitivity to pain and heat, leads to a diminished perception of pain stimuli. In addition, PN affects the motor system causing a progressive weakening of the intrinsic muscle components. Furthermore, the autonomous component of neuropathy leads to anhydrosis resulting in dry skin with lowered barrier function, and increases the arterio-venous shunting, leading to altered skin and bone perfusion.

INFLAMMATION IN WOUND HEALING

Skin injury causes the immediate onset of acute inflammation. The inflammatory response is mostly coordinated by two principal effectors derived from the circulation: polymorphonuclear neutrophils (PMNs) and monocyte-derived macrophages.

Neutrophils appear earlier and in a larger amount in the wounded area than monocytes, partly due to their greater count in circulation. Therefore, the initial inflammatory phase is characterized by a massive infiltration of PMNs. Later in inflammation, the number of PMNs declines and macrophages predominate.

PMNs and monocytes from dermal blood vessels are attracted to the site of injury by chemotatic signals, including complement factor 5a, fibrin by-products, platelet derived growth factor (PDGF), transforming growth factor-beta (TGF-β), and even microbial peptides such as lipopolysaccharides and formyl-methionyl peptides [15-17]. At this phase, mast cells play an important role on leukocyte recruitment, as they release other potent chemoattractants, such as tumor necrosis factor (TNF), histamine, proteases, leukotrienes, and cytokines [18].

As monocytes migrate to the wounded area, they differentiate into larger phagocytic macrophages. Monocyte/macrophage-specific chemotatic agents, monocyte chemoattractant protein-1 (MCP-1) [19] and macrophage inflammatory protein-1 (MIP-1) [20], are responsible for further monocyte homing.

At the wound site, neutrophils and macrophages bind to specific proteins of the extracellular matrix and phagocyte microorganisms, foreign particles and cell debris, controlling and fighting infection. At the same time, these cells also secrete specific chemoattractants, cytokines, growth factors and reactive oxygen and nitrogen species that mediate a cascade of events to induce granulation tissue formation. TNF-α, interleukin-1 (IL-1), interleukin-6 (IL-6), PDGF, transforming growth factor alpha (TGF-α), TGF-β, epidermal growth factor (EGF), basic-fibroblast growth factor (b-FGF), vascular endothelial growth factor (VEGF), and nitric oxide (NO) are some of the substances released by macrophages and/or by neutrophils that contribute to the activation, proliferation and migration of fibroblasts and ECs from the adjacent dermis [5,21,22]. Therefore, this inflammatory cell influx is crucial in the transition between the processes of inflammation and the actual repair phase. A highly regulated, self-limited inflammatory response is necessary for proper wound healing. PMNs responsiveness is controlled in time, space and magnitude by apoptosis and/or by macrophage-mediated phagocytosis.

The macrophage is classically considered the principal inflammatory cell in wound healing. Besides clearing the excessive neutrophils from the wounded area, they appear to be the main source of the growth factors cocktail that is required for granulation tissue formation. The observations that, in the absence of massive infection, wound healing is not affected in neutropenic animals, whereas animals deprived of macrophages presented a severe impairment of the wound healing machinery, gave rise to the "macrophage dogma" [23]. Other studies also support the importance of these cells in wound repair [24-26].

Nevertheless, this concept has been challenged by different evidences, including: a) the observation that wound repair in early embryos, where there is no inflammatory cell influx to the wound site, is virtually perfect [27,28]; b) the well known fact that in some dermatopathological settings of intense local accumulation of neutrophils and/or macrophages wound healing is abrogated; c) the finding that transgenic mice constitutively expressing the chemokine IP-10 and exhibiting an elevated inflammatory response show impaired granulation tissue formation and delayed wound healing [29].

Therefore, PMNs and monocytes/macrophages may be necessary for wound healing, however, under certain circumstances, skin repair may occur in their absence, or be impaired in case of an excessive response of these inflammatory cells.

DIABETES AND INFLAMMATION

A diabetes setting, either of type 1 or type 2 DM, is characterized by sustained hyperglycemia and chronic elevation of pro-inflammatory mediators. This pro-inflammatory environment induces and perpetuates the inflammatory responses, leading to a chronic inflammatory state. However, this condition is considered a low-grade inflammation, since the hyperglycemic background drives to impaired cellular defense mechanisms.

Studies have shown that in the serum of type 2 diabetes patients, many pro-inflammatory substances, such as TNF-α, IL-6 and IL-1 are elevated and have been linked to the development of insulin resistance [30,31]. In addition, analysis of the fluid of diabetic wounds from both animal models and human patients has shown insulin-degrading activity, which has in turn been correlated with the levels of haemoglobin A1c [32]. This suggests a straight relationship between hyperglycemia and the wound proteolytic environment. Also, in diabetes, neutrophils show a reduction in chemotatic and phagocytic activities, rendering the wounds more prone to infection [33,34].

As a result, and in contrast to normal wound healing, where inflammation occurs in a sequential, regulated and self-resolving manner, the immune reaction in diabetic wounds appears prolonged and non-effective.

NEOVASCULARIZATION IN WOUND HEALING

In normal mature tissues the vessels are in general quiescent as tissue cells produce low levels of pro-angiogenic molecules and high levels of angiogenesis inhibitors. In the presence of skin injury, platelets, inflammatory cells, fibroblasts and injured EC secrete angiogenic factors that trigger the formation of new blood vessels within the granulation tissue. Neovascularization of the wound's granulation tissue occurs as a result of angiogenesis and/or vasculogenesis [35].

ANGIOGENESIS

Angiogenesis is the process by which resident EC of the wound's adjacent mature vascular network, in response to angiogenic signals, proliferate, migrate, and remodel into new capillaries that grow within the wound substrate [35-39].

A vast array of molecules, including growth factors, extracellular matrix proteins (ECM), matrix metalloproteinases (MMPs), integrins and cytokines, interplay co-ordinately in highly complex scenarios to re-establish the vasculature at the injured site.

Wound-induced hypoxia (low oxygen tension) stimulates vascular regeneration by activating hypoxia-inducible transcription factors (HIF-1α), which increase the production of angiogenic growth factors such as VEGF [40,41] and the expression of the chemokine receptor CXCR4 [42]. Also, platelets secrete PDGF [43], VEGF [44], bFGF/FGF-2 [45], TGF-β [45] and angiopoietin-1 (Ang-1) [46]. Moreover, activated fibroblasts, inflammatory cells, keratinocytes and even EC are capable of producing growth factors and cytokines. Together, these molecules create a potent angiogenic signal that stimulates EC proliferation and migration [47-49]. VEGF and FGF-2 are the main stimulators of blood vessels formation. FGF-2 is believed to be an early trigger of angiogenesis, whereas VEGF seems to be responsible for the sustained neovascular growth.

Migration of EC to the wound site occurs as a result of vascular permeability, peri-endothelial support loss and disruption to the basal membrane [50]. Proteolytic enzymes, such as urokinase plasminogen activator and MMPs, are key effectors on this process [51]. MMPs digest specifically the ECM components of the provisional matrix and stimulate cell proliferation and migration, either directly through activation of EC or indirectly via interaction with adhesion molecules [51].

As EC enter the wound tissue, they must continue to proliferate, arrange three-dimensionally as channels and produce a basal lamina [52]. Fibroblasts produce ECM proteins that act as a scaffold support for the new vascular network, through which EC may migrate, and as a reservoir and modulator for growth factors such as FGF-2, VEGF and PDGF [39,53].

Finally, PDGF-BB recruits vascular smooth muscles cells (VSMCs) and pericytes to provide stabilization and maturation of the neovasculature [54-56].

VASCULOGENESIS

Vasculogenesis is the process by which EPCs are recruited from the bone-marrow to the peripheral circulation and subsequently to the wound area, differentiate into mature EC and give rise to a vascular network de novo [57-59].

Bone marrow-derived EPCs (BM-EPCs) respond primarily to local tissue ischemia by following angiogenic chemokine gradients that stimulate EPCs recruitment and subsequent homing to the site of injury, where they participate in neovasculogenesis [58,60,61]. Under the hypoxic stimuli, VEGF is released and upregulates the production of NO in the bone marrow (BM) through the activation (phosphorylation) of the stromal BM nitric oxide synthase (NOS). In this process, the endothelial isoform of nitric oxide synthase (eNOS) plays a central role [62,63]. Elevated NO levels stimulate EPC production from the BM, proliferation and mobilization to the bloodstream, whereas the chemokine stromal cell-derived factor-1α (SDF-1α)/CXCR4 axis promotes the homing of these cells to the wound area.

In addition to VEGF, placental growth factor (PlGF) also exerts its vasculogenic effects through VEGF receptor 1 (VEGF-R1) and activates MMP-9. MMP-9 stimulates the secretion of soluble kit-ligand (sKitL) from the membrane-bound mKitL on EPCs. This signal contributes to EPC proliferation, recruitment and maturation [64].

Furthermore, adhesion molecules, such as selectins and β-integrins, as well as activated platelets promote adhesion and differentiation of EPCs [65,66]. Vascular smooth muscle cells may also sequester circulating EPCs through chemokine secretion [67]. Once recruited and firmly adhered to the site of injury, EPCs contribute to the maintenance of the endothelial monolayer but also exert paracrine effects by producing angiogenic growth factors, which in turn promote mobilization of adjacent mature EC [68].

ANGIOGENESIS AND DIABETIC WOUND HEALING

The control of vascular growth has clinical importance since several disease states are associated with imbalances in blood vessel formation, such as diabetes mellitus. Patients with diabetes show abnormal angiogenesis in various organs. Vasculopathies associated with diabetes include increase in blood vessel formation (e.g. retinopathy, nephropathy) and accelerated atherosclerosis leading to coronary artery disease, peripheral vascular disease, and cerebrovascular disease [69]. However, in diabetes impaired wound healing angiogenesis is decreased [70], and this limited penetration

of new blood vessels into the wound restricts the entry of inflammatory cells. In turn, the total amount of factors released by these cells will be decreased. A number of growth factors essential for wound healing, including FGF-2 and PDGF, have also been found to be reduced in experimental diabetic wounds [71-74]. Also, Topical administration of high glucose to wounds of non-diabetic rats was shown to inhibit the normal angiogenic process [75], suggesting a direct role for high glucose levels in diminished angiogenesis in diabetes.

VEGF, which plays an important role in vascular growth, has been shown to be deficient in experimental and clinical diabetic wounds [76]. Studies in diabetic animals have shown that modulation of oxidative damage [77] or inhibition of the receptor for advanced glycation end products [78] improve wound healing and were associated with the up-regulation of endogenous VEGF. Moreover, VEGF administration improves healing in nondiabetic ischemic wounds [79] and blocking VEGF with neutralizing antibodies impedes tissue repair [80]. Together, these evidences 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 [76,81]. In fact, Galiano and collaborators [82] found that topical VEGF accelerates wound healing in a diabetic mouse model.

In conclusion, inflammation and neovascularization are essential for wound healing. However, these phenomena must be sequential, self-limited and highly controlled through orchestrated cell-molecule interactions, in order to achieve proper wound healing. In a diabetes setting, abnormal wound healing occurs as a result of a blunted acute inflammatory response and an impaired angiogenesis (Fig. **1**).

ENDOTHELIAL PROGENITOR CELLS AND DIABETIC WOUND HEALING

Angiogenesis is not the only mechanism by which new vessels are formed, as explained before. EPCs are mobilized in response to trauma or ischemia and are able to contribute to tissue repair and new blood vessel formation [58,83-85]. Increasing evidence suggest that wound-healing mechanisms, in both the bone marrow and within the skin wound, are compromised by diabetes as a result of impaired bone marrow-derived EPCs [86-91]. It was demonstrated that EPCs isolated from diabetic patients have impaired proliferation, adhesion, and incorporation into vascular structure [90]. Also, the numbers of EPCs are decreased in diabetic patients [89,90] and EPC recruitment into the wound site after vascular injury is also impaired in diabetes [92]. These alterations are likely to be involved in the pathogenesis of vascular disease in diabetes [86].

Growth factor such as VEGF can induce the release of progenitor cells from the bone marrow. However, the non-specific effects of these growth factors in other cells, such as white cells and platelets, or the leaky-capillary effect raise concerns in using these factors to treat diabetic patients with nonhealing chronic wounds [93-96].

EPCs are mobilized by eNOS activation in the bone marrow. This process is possibly impaired in diabetes since eNOS activity was shown to be decreased in diabetic mice [60], thus preventing EPCs from reaching the wound site in

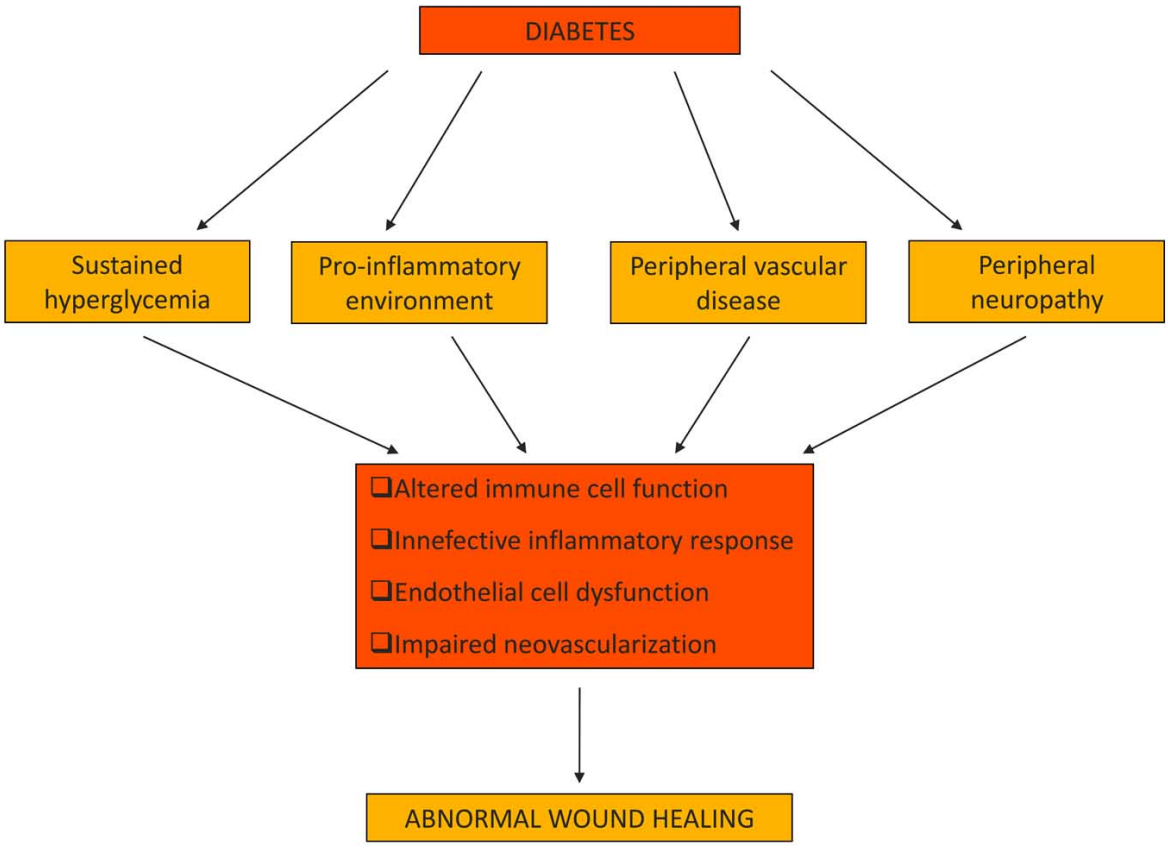

Fig. (1). Overview of diabetes impaired wound healing.

significant numbers. Hyperoxia has been shown to stimulate eNOS activation in some tissues [97]. Although the total numbers of active EPCs were much lower in diabetic mice than in controls, hyperoxia showed to increase the mobilization of EPCs from the bone marrow to the bloodstream. An increase in bone marrow eNOS activation as a result of hyperoxia leads to EPC mobilization into the bloodstream [60]. However, hyperoxia is not effective in the EPC mobilization to the wound site as this process is related with signals that attract and stimulate these cells to migrate to sites of injury. EPC recruitment to the wound site depends on ischemia-induced upregulation of SDF-1α. The expression of SDF-1α was found to be decreased in epithelial cells derived from wounds of streptozocin-induced diabetic mice [60] and this effect was found to be responsible for a decreased EPC recruitment to the wound site. More important, in combination with hyperoxia, which brings an increased amount of circulating EPCs, the local injection of recombinant SDF-1α protein significantly enhances EPC recruitment to wound tissues and improves wound healing in diabetic animals [60].

NEUROPEPTIDES AND WOUND HEALING

The peripheral nervous system (PNS), acting through neuropeptides, not only relays sensory information to the central nervous system (CNS) but also plays a role in the inflammatory, proliferative, and reparative processes after injury. Neuropeptides mediate their actions by binding to specific receptors found on various cells in the skin, including immune cells, Langerhans cells, EC, mast cells, fibroblasts and keratinocytes [98]. Several neuropeptides are involved in wound healing, including substance P (SP), neuropeptide Y (NPY) and calcitonin gene-related peptide (CGRP) [99-101] (Table **1**).

SUBSTANCE P

Substance P, an 11–amino acid peptide, is a member of a family of structurally related peptides called tachykinins. Substance P is present in many areas of the CNS and PNS. In the periphery, SP is located especially in areas of immunologic importance, such as the skin, gastrointestinal tract, and respiratory tract [102]. Substance P is synthesized in the dorsal root ganglia, from which it migrates centrally to the dorsal horn of the spinal cord and peripherally to nerve terminals of sensory neurons [103]. The tachykinins bring about their actions mainly by activating 3 primary types of receptors: NK1, NK2, and NK3. All 3 receptors are members of the superfamily of receptors coupled to G-regulatory proteins. Receptor stimulation leads to the activation of phospholipase C and thus to the generation of inositol triphosphate and diacylglycerol and to the release of Ca^{2+} from internal stores [104-106].

ALTERATION OF SUBSTANCE P IN DIABETES

Morphological and immunohistochemical studies in type 1 diabetic subjects have shown a depletion of SP in the central and peripheral nervous system, especially in those with diabetic neuropathy [107]. Also, the number of SP-positive fibers are decreased in the dermis of diabetic patients with diabetic neuropathy [108]. Moreover, Gibran and collaborators [109] have demonstrated that exogenous SP improves wound healing kinetics in diabetic db/db mice and they have also demonstrated fewer nerves in the epidermis of skin from diabetic patients and from db/db mice. These results suggest that insufficient nerve-derived mediators such SP contribute to the impaired response to injury.

The biological action of substance P is regulated by a cell surface metallopeptidase, neutral endopeptidase (NEP), which degrades SP. The skin of diabetic patients with diabetic neuropathy was found to have an elevated NEP activity that may contribute to deficient neuroinflammatory signaling and impair wound healing [110]. Also, the skin from diabetic db/db mice have an increased NEP activity and NEP inhibition was found to improve wound closure kinetics [111].

SUBSTANCE P CONTRIBUTION TO INFLAMMATION AND ANGIOGENESIS IN WOUND HEALING

Substance P is released by the sensory nerve fibers during tissue insult and it modulates responses in the skin by activating a number of target cells via neurokinin receptors. Substance P and other tachykinins are able to cause vasodilation because of direct actions on vascular smooth muscle and enhanced production of nitric oxide by the endothelium [112,113]. In addition, SP can initiate increased vascular permeability and protein extravasation after tissue injury [114,115], enhancing leukocyte infiltration to tissues [102]. Also, SP acts as a strong chemoattractant for immune cells. SP can stimulate the production of TNF-α, IL-1β, IL-2, IL-8 and IL-6 from T cells, macrophages and neutrophils [116] and it modulates leukocyte adhesion to microvascular EC in the skin [117] by the upregulation of cell adhesion molecules, such as intracellular adhesion molecule (ICAM)-1, vascular cell adhesion molecule (VCAM)-1, P-selectin, leukocyte-function-associated antigen (LFA)-1 in EC and lymphocytes, essentially through intracellular Ca^{2+} mobilization

Table 1. Effect of Neuropeptides on Cutaneous Wound Healing (Adapted from Pradhan and Colleagues [176])

Neuropeptide	Factors Involved	Function	Involved in which Wound Healing Phase
Substance P (SP)	TNF-α, IL-1b, IL-2, IL-8, IL-6, TGF-β, ICAM-1, VCAM-1, LFA-1, Nitric oxide	Vasodilation, vascular permeability, leukocyte attraction and adhesion, cell proliferation and migration	Inflammation Angiogenesis
Neuropeptide Y (NPY)	IL-2 and TNF-α	Migration of macrophages Cell proliferation	Inflammation Angiogenesis
Calcitonin gene-related peptide (CGRP)	IL-1α, IL-1β, IL-8, IL-2, IL-6, TNF-α, VEGF	Vasodilation, vascular permeability, cell proliferation, acts in combination with SP	Needs investigation

and NF-AT- and NF-κB pathways [118-123]. The leukocytes that extravasate to the tissues after injury will promote a proinflammatory microenvironment that leads to proliferation of EC and angiogenesis.

Results show that SP can have a trophic function in wound healing by the stimulation of angiogenesis. SP was shown to mediate wound healing, after UV-induced damage of the skin, by stimulation of angiogenesis or epidermal cell proliferation [124]. Moreover, substance P stimulated angiogenesis in an *in vitro* model using human umbilical cord vein EC cultured on a basement membrane (Matrigel) substrate and it stimulates EC differentiation into capillary-like structures in a dose-dependent manner [117]. Capillary and fibroblast proliferation were found to be accelerated by SP after operative repair of the Achilles tendon in rats [125]. In addition, elevated glucose and fatty acid levels as seen with diabetes mellitus, were found to inhibit SP mediated human microvascular endothelial cell (HMEC-1) directional migration and proliferation [126].

Substance P plays a major role in the inflammatory and angiogenesis phases of wound healing, and dysregulation of the SP pathway in diabetes can impair wound healing.

NEUROPEPTIDE Y

NPY is a highly conserved polypeptide composed of 36 aminoacids [127] and it is widely present in the CNS and PNS. NPY regulates multiple physiological processes including hypothalamic control of food intake, anxiety [128], and modulate the immune function [129]. NPY is not only expressed in the nervous system, but also by the heart, immune cells and EC [130-134]. The NPY acts trough NPY receptors. The cDNAs of four NPY receptors have been cloned: Y1, Y2, Y4 and Y5. These receptors mediate their responses through G proteins, resulting in inhibition of adenylate cyclase activity and increase in intracellular [Ca^{2+}] [135]. NPY regulates vasoconstriction and stimulates vascular smooth muscle cell proliferation [136]. Also, it inhibits T cell activation modulating the inflammatory signal [137]. Moreover, NPY stimulates proliferation, migration and formation of EC [138].

ALTERATION OF NEUROPEPTIDE Y IN DIABETES

The levels of NPY in the CNS, particularly in the hypothalamus, were found to be increased in diabetic patients [139], however, in the skin, the NPY levels showed to be reduced in diabetic patients [140-142]. Also, the expression of NPY was shown to be reduced in diabetic rats [143]. Moreover, it was shown a diminished contractile response to NPY of arteries from diabetic rabbits [144].

NEUROPEPTIDE Y CONTRIBUTION TO INFLAMMATION AND ANGIOGENESIS IN WOUND HEALING

NPY has pleiotropic effects on both the innate and adaptive immune system, acting through Y1 receptor, with effects ranging from the modulation of cell migration to macrophage, T helper (Th) cell cytokine release, and antibody production [145]. NPY was shown to stimulate proliferation and migration of EC [146,147]. In HUVECs, NPY was shown to be co-localized with dipeptidyl peptidase IV (DPPIV) which cleaves NPY(1-36) to form NPY(3-36) resulting in the formation of a non-Y1 receptor agonist, which remains angiogenic. The blockage of DPPIV by using monoclonal antibodies showed to decrease the migration of HUVECs in response to NPY(1-36) or NPY(3-36) following cell wounding. These results suggest that non-Y1 receptors activated by NPY(3-36) mediate angiogenic effects of NPY [146]. Also, Y2 receptor and DPPIV expression was significantly reduced in ageing mice [148]. Moreover, mice with deleted Y2 receptor showed to have a delayed wound healing caused by the blockage of NPY-induced angiogenesis [100]. In conclusion, NPY is also important for both the inflammatory and angiogenic phases of wound healing.

CALCITONIN GENE-RELATED PEPTIDE

Calcitonin gene-related peptide, a 37–amino acid peptide, is known to be generated from the alternative splicing of the calcitonin gene, both in the CNS and the PNS [149]. CGRP is released together with SP from peripheral sensory nerves [150,151]. The CGRP receptors were shown to be present in the brain and also in other organs such as heart, vasculature, liver, spleen, skeletal muscle and lung, and in lymphocytes [149]. Pharmacologically, a two CGRP1 and CGRP2 receptor subtypes has been proposed [152]. Stimulation of CGRP receptors in various cells and tissue has been shown to increase intracellular cyclic adenosine monophosphate (cAMP) and to activate adenylate cyclase [149]. Peripheral secretion of CGRP causes prolonged increases in blood flow [153]. Unlike SP, CGRP is not capable of enhancing vascular permeability on its own but potentiates the protein extravasation induced by tachykinins [154,155].

ALTERATION OF CGRP IN DIABETES

CGRP is reduced in the heart, dorsal root ganglion and in primary sensory neurons of diabetic animal models [156-158]. Also, both CGRP and CGRP receptor expression were shown to be reduced in a diabetic cardiomyopathy model [159,160]. Moreover, the function of CGRP is altered in diabetes and recent studies have shown that CGRP-mediated vasodilation is significantly reduced in diabetic rats [161-163].

CGRP CONTRIBUTION TO INFLAMMATION AND ANGIOGENESIS IN WOUND HEALING

CGRP is known to induce neurogenic inflammation, inducing pro-inflammatory response in several cell types, and it also induces the formation of new vessels, important during physiological and pathophysiological wound healing [101,164]. CGRP induces inflammatory pathways but also inflammation can induce CGRP release [165,166]. The pro-inflammatory action of CGRP includes the stimulation of the adhesion of immune cells to EC [167], the induction of vasodilation, plasma extravasation, and the expression of adhesion molecules on vascular EC [167-169]. CGRP also regulates cytokine production. Keratinocytes were shown to increase the release of IL-1α and IL-8 induced by CGRP [170]. CGRP also inhibits IL-2 production from T lymphocytes [171] and increases, in combination with SP, the levels of IL-1β, IL-6 and TNF-α in human dental pulp fibroblasts and IL-1β and TNF-α in macrophages [172,173]. CGRP can also stimulate the proliferation of keratinocytes, EC and melanocytes [164,174,175]. In CGRP knockout mice (CGRP-/-), angiogenesis and wound healing were impaired

compared with those in wild-type mice, and a decrease in the release of VEGF from the wound granulation tissues was shown [101]. This suggests that the involvement of CGRP in wound healing is mediated through its effect on angiogenesis. Therefore, CGRP derived from neuronal systems may facilitate wound healing and angiogenesis.

FUTURE THERAPIES

Growth Factors

The complex sequences of molecular and cellular events that are carefully orchestrated during wound healing are difficult to reproduce. Nevertheless, administration of growth factors has been shown to promote healing by affecting all phases of wound healing. Several growth factors have been studied for the promotion of diabetic wound healing, such as FGF [177-179], VEGF [48,82], granulocyte colony-stimulating factor [180-182], and hepatocyte growth factor [175,183-187]. Despite high expectations in laboratory models of wound healing, growth factor delivery has shown to have a limited clinical success. However, PDGF-BB was shown to be an exception being successful in promoting wound healing in clinical trials, resulting in the first growth factor delivery product, Regranex®, approved by the Food and Drug Administration for treatment of diabetic foot ulcers [188,189]. The difficulty other growth factors in being successful can be attributed, at least in part, to their short half-live [190], which is due to the rapid degradation in the protease-rich environment of the wound leading to a decrease bioactivity [191]. Another reason for le lack of therapeutic effect may be correlated with the use of single growth factors. The judicious combination of several growth factors or with other wound healing therapeutic strategies may be necessary to achieve better clinical results.

Gene Therapy

The skin has become an important target of gene therapy research because it is accessible and the effects of therapy can be easily monitored. Also, this research was made possible due to the harvest and cultivation ease of fibroblast and keratinocyte, thus allowing *in vitro* testing for gene transfer and the use of skin cells as vehicles in gene transfer [192]. Gene delivery has also been explored as an alternative to growth factor delivery because infiltrating cells uptake the genes and produce continuously the therapeutic protein(s) in the local environment [193,194].

Several methods of gene delivery have been studied. Viral vectors are the original and most established technology for gene delivery, including adenovirus and lentivirus. A wide range of applications have been developed and many virus-mediated gene transfer models are successful. The production of viral vectors, however, is time and cost consuming, transfection efficacy is variable, and the risk of local or systemic infections remains a concern. Non-viral gene therapy, which is performed without a viral vector, includes the use of naked DNA, liposomes, electroporation or biomaterials. This approach can eliminate the risk of infection and cost of vector production. The transient nature of gene expression is also a benefit in wound healing applications.

Adenoviral delivery of PDGF-B has been shown to significantly enhance wound closure, granulation tissue formation and vessel density in db/db, streptozotocin, and non-obese diabetic (NOD) mice [87]. Interestingly, lentiviral delivery of the gene encoding PDGF-B also enhanced angiogenesis and collagen deposition in diabetic wounds but did not affect re-epithelialization [195]. Adenoviral delivery of Ang-1 was also shown to improve wound repair in diabetic mice by inducing angiogenesis in a VEGF-independent manner [196]. Moreover, lentiviral-mediated transfection of SDF-1α in diabetic mice also improve wound healing [197]. The use of a fibrin scaffold was shown to provide an enhanced method of gene transfer of adenovirus encoding eNOS, compared with direct delivery of adenovirus encoding NOS alone and fibrin scaffold alone. This is due to the ability of fibrin to retain the vector at the wound site, and present the adenovirus to infiltrating cells involved in wound healing. The eNOS gene delivery showed to increase nitric oxide production and improve wound healing, as shown by improving epithelialization, maturation of angiogenesis and inflammatory response [198].

Electroporation has been successfully used to accelerate the closure of diabetic wounds [199]. Also, it was shown that the synergistic use of electroporation, where an electric field is applied to tissue, in combination with TGF-β1 cDNA improves wound healing, in a diabetic mouse model, showing an increased rate of re-epithelialization, angiogenesis, and collagen synthesis [200]. Moreover, the electroporation and simultaneous administration of keratinocyte growth factor (KGF) plasmid DNA showed to increase wound healing [201].

These techniques need further investigation to define their efficacy and clinical applicability. Also, it is important to study the growth factor levels in different phases of wound healing and to elucidate the precise timing of gene expression or downregulation required to better improve wound healing.

CELL-BASED THERAPIES

Cell-based therapy is a promising therapeutic option for treating patients with diabetic, nonhealing wounds. Several studies have investigated the potential of stem cells, keratinocytes, and fibroblasts for the treatment of chronic wounds. However, little is known about the use of most of these cells in diabetic wounds, and the only FDA-approved treatments include fibroblasts delivered in an absorbable mesh [202], and fibroblasts and keratinocytes delivered in type 1 collagen [203].

The use of stem cells in diabetic wounds has been studied only in animal models with relative efficacy. Bone-marrow mesenchymal stem cells (BM-MSC) are capable of self-renewing and of differentiating into various tissues and cells, such as liver epithelium, lung, gastrointestinal tract, and skin cells [204,205]. In fact it was shown that allogeneic BM-MSCs significantly enhanced wound healing in normal and diabetic mice through differentiation and release of proangiogenic factors [206]. Moreover, CD34+ cells were shown to improve the healing of diabetic wounds with an increase in revascularization [207]. EPCs are one type of cells that have been moved from experimental models to clinical trials. EPCs seem to be ideal candidates for *in vivo* cell based therapies for ischemia. However, EPCs from diabetic patients have been shown to be dysfunctional with impaired proliferation, adhesion, and incorporation into vascular struc-

tures, thus, autologous EPCs could be of limited use [90,208]. The efficacy of cell therapies to augment neovascularization and healing depends not only on the sufficient amount of circulating EPCs, but also on the efficient recruitment of these cells to the target tissue. Therapeutic interventions, including correcting both EPC activation via hyperoxia and EPC homing via administration of SDF-1α, may significantly accelerate diabetic wound healing by correcting the EPC deficit inherent to diabetic wounds. If simultaneously combined with current therapies, and with other potential treatments targeting eNOS activation and EPC recruitment, might further stimulate healing. A better understanding of the molecular and cellular mechanisms of nonhealing diabetic wounds in combination with development of efficient approaches for correcting EPC deficits and functional impairments will potentially result in the development of efficient therapies that prevent wound progression, eliminate amputations, and promote rapid healing in patients with diabetes.

NEUROPEPTIDES

The immune system is normally regarded as self regulating, but several studies suggest that the peripheral nervous system closely interacts with the immune system and that it plays important neuroimmuno regulatory functions [209,210]. Afferent nerves can respond to a large range of stimuli and, upon stimulation, neuropeptides are rapidly released in the microenvironment [209,210]. In skin, these neuropeptides act through the activation of receptors present in a variety of cells, including microvascular EC, keratinocytes, mast cells, fibroblasts, and immune cells. Impaired neurogenic inflammation, due to diabetic neuropathy, contributes to enhanced susceptibility for diabetic foot ulcer. The neuropeptides known to be involved in impaired diabetic wound healing are substance P [99,211] and neuropeptide Y [100,212]. Further research is needed in order to evaluate the effect of the addition of neuropeptides to impaired wound healing and possibly consider this approach as a future therapy.

CONCLUSION

Despite the studies on chronic diabetic wounds, the problem persists and the success of currents treatments is limited. The complex molecular events that underlie successful wound healing limits the effectiveness of the treatments and thus monotherapy is not the better option. Novel discoveries of disease molecular pathogenesis from studies of patient biopsies and animal models by using molecular analyses, such as genomics and proteomics, and the use of transgenic mice, will lead to a better understanding of deficient wound healing in diabetes. These new findings with the development of systems for sustained topical delivery (such as polymers, gene delivery systems), and major advances in tissue engineering (such as human skin engineering, cellular matrices, and cell-based therapies), hold the promise of the potential application of these technologies to people with diabetic wounds in the near future. Eventually, the development of a combination of treatments that ensures rapid and complete healing is the key strategy for the management of chronic nonhealing diabetic wounds.

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