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Invited review

Redox signals in wound healing

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Abstract

Physical trauma represents one of the most primitive challenges that threatened survival. Healing a problem wound requires a multi-faceted comprehensive approach. First and foremost, the wound environment will have to be made receptive to therapies. Second, the appropriate therapeutic regimen needs to be identified and provided while managing systemic limitations that could secondarily limit the healing response. Unfortunately, most current solutions seem to aim at designing therapeutic regimen with little or no consideration of the specific details of the wound environment and systemic limitations. One factor that is centrally important in making the wound environment receptive is correction of wound hypoxia. Recent work have identified that oxygen is not only required to disinfect wounds and fuel healing but that oxygen-dependent redox-sensitive signaling processes represent an integral component of the healing cascade. Over a decade ago, it was proposed that in biological systems oxidants are not necessarily always the triggers for oxidative damage and that oxidants such as H_2O_2 could actually serve as signaling messengers and drive several aspects of cellular signaling. Today, that concept is much more developed and mature. Evidence supporting the role of oxidants such as H_2O_2 as signaling messenger is compelling. A complete understanding of the continuum between the classical and emergent roles of oxygen requires a thorough consideration of current concepts in redox biology. The objective of this review is to describe our current understanding of how redox-sensitive processes may drive dermal tissue repair.

Keywords: Tissue repair; Oxygen; Oxidants; Hydrogen peroxide

Physical trauma represents one of the most primitive challenges that threatened survival. In other words, injury eliminated the unfit. Ancient scriptures depicting the science of life or *Ayurveda* report refined clinical surgical procedures such as rhinoplasty and cheek flaps as early as in 6th–7th century BC. This was the beginning of planned physical injury with the intent to cure [1,2]. Today, surgical trauma taken together with injury caused during accidents and secondary to other clinical conditions *e.g.* diabetes represent a substantial cost to society [3,4]. The search for therapeutic strategies to manage wounds, especially chronic, led to numerous solutions the vast majority of which failed to deliver clinically [5,6]. For example, the sharp rise in enthusiasm for growth factors met disappointing clinical outcomes [6–10]. On a brighter note, we did learn that any solution to wound healing will require a multi-faceted comprehensive approach. First and foremost, the wound environment will have to be made receptive to therapies. Second, the appropriate therapeutic regimen needs to be identified and provided while managing systemic limitations that could secondarily limit the healing response. Unfortunately, most current solutions seem to aim at designing therapeutic regimen with little or no consideration of the specific details of the wound environment and systemic limitations. In order to make the wound environment receptive, a few key considerations exist. For example, surgical debridement must remove excess burden of dead and diseased tissue where applicable. The wound needs to be kept moist and warm. Excessive infection needs to be managed. All clinical wounds are expected to carry some microbial load. When the load is high causing overt infection, among other things it places a high demand on oxygen supply because O_2-dependent respiratory burst represents a primary mechanism to endogenously limit wound infection. In addition, the injured tissue needs to fuel the reparative process by means of oxidative metabolism

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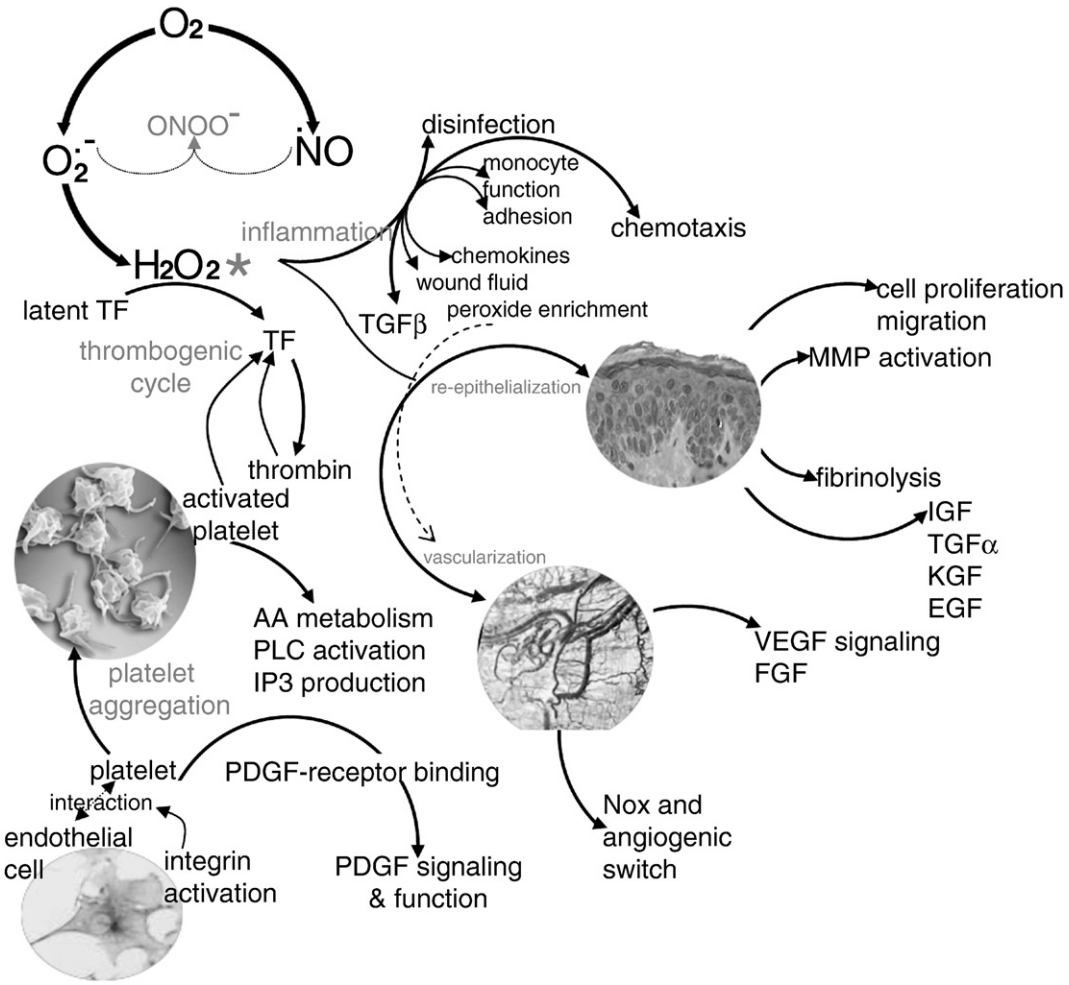

Fig. 1. Overview of the major redox-sensitive events in cutaneous wound healing. See text for details.

and thus requires additional oxygen. These amplified burdens of oxygen demand on the injured tissue which characteristically suffers from disrupted vasculature leads to oxygen deficit causing wound hypoxia. Wound hypoxia limits healing and the state of tissue oxygenation is an important determinant of successful healing. Recent work have identified that oxygen is not only required to disinfect wounds and fuel healing but that oxygen-dependent redox-sensitive signaling processes represent an integral component of the healing cascade. The widely held notion that biological free radicals are necessarily agents of destruction is now facing serious challenge [11]. Over a decade ago, it was proposed that in biological systems oxidants are not necessarily always the triggers for oxidative damage and that oxidants such as H_2O_2 could actually serve as signaling messengers and drive several aspects of cellular signaling [12]. Today, that concept is much more developed and mature. Evidence supporting the role of oxidants such as H_2O_2 as signaling messenger is compelling [13–23]. A complete understanding of the continuum between the classical and emergent roles of oxygen requires translational research that would have to span between the molecule to the man and back and much depends on the rapidly unfolding science of redox biology. The objective of this review is to describe our current understanding of how redox-sensitive processes may drive dermal tissue repair (Fig. 1). The field of redox control of wound healing is in its nascent stage. On one hand, the literature reviewed herein presents a compelling case for why wound scientists should care more about the redox biology of wounds. On the other hand, this article seeks to draw the interest of basic redox scientists towards a novel and lucrative translational application potential *i.e.* redox-based wound therapeutics.

1. Hemostasis

As the first step in response to injury, blood flow around the injured site increases helping to bring in blood-borne products such as the white blood cells, antibacterial proteins and other relevant products as first-aid. ROS and the redox state are important in the control of blood coagulation and thrombosis [24]. Furthermore, vascular injury triggers endothelial exocytosis of granules, releasing pro-inflammatory and pro-thrombotic mediators into the blood by redox-sensitive mechanisms [25]. The onset of hemostasis is caused by the tissue factor (TF) which initiates the extrinsic coagulation cascade leading to thrombin formation. In turn, thrombin induces TF mRNA in vascular smooth muscle cells (VSMCs), thereby contributing to the

prolonged pro-coagulant activity and enhanced thrombogenicity at sites of vascular injury. Thrombin can also interact with the vascular wall via specific receptors and can increase vascular TF expression. Reactive oxygen species (ROS) and the ROS-generating NADPH oxidases play important roles as signaling molecules in the vasculature [26,27]. H_2O_2 activates latent cell surface tissue factor [28]. Surface exposure of active TF leads to the formation of the TF/VIIa complex, which promotes the generation of thrombin. Thrombin activates ROS generation by vascular NADPH oxidases, subsequently initiating and activating ROS-dependent signaling cascades that promote a thrombogenic cycle via up-regulation of TF. This cycle is further fueled by the sustained generation of ROS, explaining the occurrence of a pro-thrombotic state. Allosteric disulfide bonds control protein function by mediating conformational change when they undergo reduction or oxidation. The known allosteric disulfide bonds are characterized by a particular bond geometry, the -RHStaple. A number of thrombosis and thrombolysis proteins contain one or more disulfide bonds of this type. TF was the first hemostasis protein shown to be controlled by an allosteric disulfide bond, the Cys186–Cys209 bond in the membrane-proximal fibronectin type III domain. TF exists in three forms on the cell surface: a cryptic form that is inert, a coagulant form that rapidly binds factor VIIa to initiate coagulation, and a signaling form that binds FVIIa and cleaves protease-activated receptor 2, which functions in inflammation, tumor progression and angiogenesis. Reduction and oxidation of the Cys186–Cys209 disulfide bond is central to the transition between the three forms of TF. The redox state of the bond appears to be controlled by protein disulfide isomerase and NO. Plasmin(ogen), vitronectin, glycoprotein 1bα, integrin beta(3) and thrombomodulin also contain -RHStaple disulfides, and there is circumstantial evidence that the function of these proteins may involve cleavage/formation of these disulfide bonds [29]. Recently it has been demonstrated that disulfide isomerization switches TF from coagulation to cell signaling [30].

Platelet aggregation and activation is another key player in re-establishing the hemostatic plug as well as in delivering facilitators of healing such as platelet-derived growth factor. Sulfhydryl and disulfide metabolism in platelet function has recently reemerged as a focus of platelet research. The wound environment is highly rich in oxidants [31] which may modify platelet function [32,33]. Platelets themselves generate ROS [34] and at the wound site co-localize with other ROS-generating cells such as white blood cells. Activated platelets up-regulate TF expression and this response involves ROS generation and a p22phox-containing NADPH oxidase in VSMC [35]. Collagen-induced platelet aggregation is associated with production of H_2O_2, which is abolished by catalase, an enzyme that destroys H_2O_2. Catalase dose-dependently inhibits thromboxane A2 production, release of arachidonic acid from platelet membrane, and inositol 1,4,5P3 (IP3) formation. In platelets stimulated with high concentrations of collagen, catalase inhibits platelet aggregation, calcium mobilization, and IP3 production. Collagen-induced platelet aggregation is associated with a burst of H_2O_2 that acts as a second messenger by stimulating the arachidonic acid metabolism and phospholipase C pathway [36]. In addition, collagen activation induces NADPH oxidase-dependent $O_2^{\cdot-}$ release in platelets, which in turn enhances availability of released ADP, resulting in increased platelet recruitment [37].

Platelet-derived growth factor (PDGF) is a family of dimeric isoforms that stimulates growth, chemotaxis and cell shape changes of various connective tissue cell types and other wound-related cells. Becaplermin (PDGF-BB), the only growth factor approved by the Food and Drug Administration, requires daily application for neuropathic wound healing. Becaplermin (0.01% Regranex gel) is a homodimeric protein produced by recombinant DNA technology through the insertion of the gene for the B chain PDGF into the yeast *Saccharomyces cerevisiae*. PDGF is dependent on H_2O_2 for its biological function [38]. The biological activity of becaplermin is similar to that of indigenous PDGF-1, specifically, the promotion of chemotactic recruitment and the proliferation of cells involved in wound repair. Engagement of PDGF with its receptor results in the generation of H_2O_2 in non-phagocytic cells by a PI3K and Rac1 dependent pathway [39]. PDGF-stimulated $O_2^{\cdot-}$ production modulates activation of transcription factor NF-kB and expression of monocyte chemoattractant protein 1 (MCP1) in human aortic smooth muscle cells [40]. Redox regulation of PDGF receptor tyrosine autophosphorylation and its signaling are related to NADPH oxidase activity through protein kinase C (PKC) and phosphoinositide-3-kinase (PI3K) activation and H_2O_2 production. Upon PDGF stimulation, PKC, PI3K and NADPH oxidase activity contribute to complete c-Src kinase activation, thus promoting maximal phosphorylation and activation of PDGF receptor tyrosine phosphorylation [41].

Glutaredoxin (GRX) is a glutathione-disulfide oxidoreductase involved in various cellular functions, including the redox-dependent regulation of certain integral proteins. GRX plays an important role in PDGF-BB-dependent cell proliferation by regulating the redox state of low molecular weight protein-tyrosine phosphatase (LMW-PTP) [42]. LMW-PTP represents a redox-sensitive protein during both platelet-derived growth factor signaling. In response to oxidation, the phosphatase undergoes a reversible inactivation, which in turn leads to the increase in tyrosine phosphorylation of its substrates. Exogenous oxidants enhance LMW-PTP tyrosine phosphorylation, through oxidation/inactivation of the enzyme, thus preventing its auto-dephosphorylation activity. Oxidants induce selective hyper-phosphorylation of Tyr132 that acts as a docking site for the adaptor protein Grb2. The redox-dependent enhancement of Grb2 recruitment to LMW-PTP ultimately leads to an improvement of ERK activation, likely triggering a prosurvival signal against the oxidant environment [43].

Platelets contain several glycoprotein receptors including the adhesion receptor glycoprotein Ib and the fibrinogen receptor glycoprotein IIbIIIa, also known as the αIIbβIIIa integrin. Both of these receptors contain thiol groups and vicinal thiols representing redox-sensitive sites. Disulfide isomerases such as protein disulfide isomerase (PDI) that are on or recruited to the platelet surface have a role in platelet aggregation. Dynamic rearrangement of disulfide bonds in receptor signaling and platelet activation is a developing concept that requires an

attacking thiol. Biochemically, a role for disulfide isomerization is suggested as the alphaIIb betaIIIa integrin undergoes major structural changes upon activation centered around a disulfide knot in the integrin. Additionally, the P2Y12 ADP receptor is involved in platelet activation by most platelet agonists and contains extracellular thiols, making it a possible site for redox modification of platelet aggregation. Various forms of redox modulation of thiols or disulfides in platelet glycoproteins exist. These include modification by low molecular weight thiols such as reduced glutathione or homocysteine, oxidized glutathione or by nitric oxide (NO) derived from *S*-nitrosothiols. Levels of these redox compounds change in various disease states and in some cases physiologic concentrations of these compounds have been shown to modify platelet responsiveness. Additionally, platelets themselves contain a transplasma membrane redox system capable of reducing extracellular disulfide bonds [32].

Integrin regulation and signaling play a central role in the hemostasis process, particularly at the level of endothelial cells by regulating the contractility and barrier function of these cells and in platelets by controlling adhesion and aggregation at the site of cell injury. Reactive oxygen species (ROS) have emerged as an important mediator both transducing the signals associated with integrin activation and modulating integrin function. Ligation of integrins in endothelial cells and platelets induces activation of the Ras/mitogen-activated protein kinase, nuclear factor-kappaB, and phosphatidylinositol 3-kinase and Rho GTPases pathways. Following vessel-wall injury and associated with activation and recruitment of platelets, there is a production of ROS concomitant with the stimulation of the blood coagulation. Moreover, ROS are capable of inducing conformational changes in integrins to change their binding affinity and function. ROS have emerged as an important modulator of integrins in coagulation through both outside-in (integrins stimulating ROS production to effect intracellular events) and inside-out signaling (intracellular ROS altering integrin function) [44].

2. Inflammation

H_2O_2 has a fine-tuning regulatory role, comprising both a pro-inflammatory control loop that increases pathogen removal and an anti-inflammatory control loop, which avoids an exacerbated harmful inflammatory response [45]. Phagocytes contribute to innate immunity by mounting a respiratory burst that helps kill internalized bacteria. Neutrophils infiltrating to the wound site cleanse the wound of foreign particles and bacteria and are then extruded with the eschar or phagocytosed by macrophages. ROS generated by both neutrophils as well as macrophages play a central role in conferring resistance to wound infection [46,47]. Blood coagulation, activated complement pathways and activated parenchymal cells at the wound site generate numerous vasoactive mediators. Experiments studying the directed locomotion of mouse peritoneal neutrophils show that at low μM concentrations H_2O_2 induce neutrophil chemotaxis [48]. Over-expression of thioredoxin, a ROS decomposing protein thiol, suppresses leukocyte recruitment induced by the murine chemokines KC/GROalpha, RANTES (regulated upon activation, normal T cell expressed and secreted), and MCP-1 [49].

Monocytes are recruited to the wound site by specific chemoattractants such as fragmented extracellular matrix protein, transforming growth factor β (TGFβ), MCP-1, and macrophage inflammatory protein (MIP). ROS induce MIP1a, MIP2 as well as MCP1 [50–52]. ROS induce TGFβ expression as well as its activation by oxidatively displacing the latency conferring peptide [53]. In certain cell types, H_2O_2 is a requirement for TGFβ-induced cell signaling [54].

H_2O_2 directly regulates monocyte function [55]. High mobility group box 1 (HMGB1) can be actively secreted by macrophages/monocytes in response to exogenous and endogenous inflammatory stimuli (such as bacterial endotoxin, TNF-alpha, IL-1, and IFN-gamma) or passively released by necrotic cells and mediates innate and adaptive inflammatory responses to infection and injury. At doses found in the wound fluid, H_2O_2 induces HMGB1 cytoplasmic translocation and active release within 3–24 h. Inhibitors specific for the JNK (SP600125) and MEK (PD98059), but not p38 MAPK (SB203580), abrogate H_2O_2 -induced, active HMGB1 release suggesting a key role of H_2O_2 in inducing active HMGB1 release, potentially through a MAPK- and CRM1-dependent mechanism [56]. Monocytes adhere to specific proteins of the extracellular matrix by their integrin receptors. Such adhesion triggers the differentiation of monocytes to reparative macrophages and stimulates phagocytosis of micro-organisms and fragments of extracellular matrix. H_2O_2 induces LFA-1-dependent neutrophil adherence and Mac-1 dependent macrophage adherence [57]. The ROS decomposing antioxidant *N*-acetyl-L-cysteine suppresses constitutive expression of CD11a/LFA-1alpha protein in cells of myeloid lineage [58]. H_2O_2 modulates leukocyte adhesion molecule expression and leukocyte endothelial adhesion [59]. Adherence of monocytes to the extracellular matrix also induces the expression of monocyte colony-stimulating factor 1 (MCSF1), a cytokine that supports monocyte and macrophage survival at the wound site. H_2O_2 is known to mediate the transcriptional induction of macrophage colony-stimulating factor [60]. Other macrophage-derived cytokines expected at the wound site include tumor necrosis factor α (TNFα) and PDGF. As discussed above, PDGF function is subject to redox control at multiple levels. TNFα biosynthesis has been shown to be ROS-inducible as well [61,62]. Several chemokine and their receptors respond to ROS. For example, ROS induce CCR5 and CXCR4 mRNA expression. CCR2, CCR5, and CXCR4 mRNA expression is sensitive to low concentrations of H_2O_2. H_2O_2 increased cell migration (3-fold) in response to MIP1 [63]. Inducible IL-1b and IL-6 expression is sensitive to ROS as well [61]. Cultured macrophages exhibit spreading in response to external stimuli. Such spreading is relevant to morphologic changes of macrophages *in vivo* during extravasation, migration, and differentiation. ROS induce spreading of macrophages via the MAP kinase-SRE signaling pathways [64].

3. Re-epithelialization

Wound closure of epithelial tissues must occur efficiently to restore rapidly their barrier function. Adult epidermal keratinocytes migrate by crawling, a process that requires protrusion of the

plasma membrane at the front of the cell and contraction of the cell body at the rear. Insulin-like growth factor 1 (IGF-1) stimulates membrane protrusion and facilitates cell spreading via activation of Rho family proteins [65]. IGF structure and function is subject to tight redox control [66,67]. Wound re-epithelialization and keratinocyte migration require strictly ordered gene expression, which is assumed to be initiated by locally released mitogens and exposure of the cells to different matrix components. Re-epithelialization of wounds involves the formation of peripheral cytoplasmic actin filaments which allow cell motility. Specific antioxidant defense mechanisms are dedicated to defend epithelial cells against excessive oxidants in the wound milieu [68,69]. At lower concentrations, ROS induce smooth muscle as well as epithelial cell proliferation and migration [70,71]. Specifically, H_2O_2 has been shown to induce pro-MMP-2 activation and cell motility [72]. Furthermore, an essential role of PKCζ in transducing a motility signal induced by superoxide has been recently demonstrated [73]. Up-regulation of ROS mediates two key events in Ras-induced morphological transformation and cell motility: it is responsible for Rac1 activation and is necessary (though insufficient) for Ras-induced cofilin dephosphorylation [74].

Fibrinolytic mechanism that comprises the activation of plasminogen into plasmin prevents excessive fibrin accumulation by promoting local dissolution of thrombi and promoting wound healing by re-establishment of blood flow [75]. During healing of skin wounds, the migrating leading-edge keratinocytes express urokinase-type plasminogen activator and its receptor [76]. $^1O_2$ converts fibrin to a form that stimulates the activation of plasminogen (bound to oxidized fibrin) by pro-urokinase and that of pro-urokinase by plasmin. The oxidative modification of fibrin by $^1O_2$ is specific and favors subsequent fibrinolysis [77]. Reactive nitrogen species, on the other hand, can cause fibrinogen nitration and may lead to a pro-thrombotic state via acceleration in formation of fibrin clots [78]. The degradation of extracellular matrix, required to allow motile wound-related cells to migrate, depends on the production of collagenase by epidermal cells as well as on fibrinolysis. Plasminogen activator also activates the collagenase MMP-1 and therefore facilitates degradation of extracellular matrix proteins thus allowing wound-cells to migrate. Inducible MMP-1 is expressed through a Nox4-mediated, ROS-dependent pathway [79]. H_2O_2 has been identified to mediate AP-1-dependent induction of MMP-1 [80]. Redox-dependent MMP-1 expression is regulated by JNK through Ets and AP-1 promoter motifs [81]. One to two days after injury, epidermal cells at the wound margin begin to proliferate behind the actively migrating cells. Epidermal growth factor (EGF), TGFα and keratinocytes growth factor support this process. H_2O_2 plays a central role in triggering EGF receptor phosphorylation and signaling [82,83]. ROS are also known to induce TGFα in fibroblasts [84]. Another significant contributor to epidermal regeneration is keratinocyte growth factor (KGF) [85]. ROS are capable of triggering KGF receptor activation and internalization, similar to those induced by KGF [86].

4. Vascularization

Vascularization, under physiological or pathophysiological conditions, typically takes place by one or more of the following processes: angiogenesis, vasculogenesis, arteriogenesis and lymphangiogenesis. Angiogenesis refers to the process by which new blood vessels develop from pre-existing blood vessels by sprouting or intussusception. *De novo* formation of new blood vessels from primitive cells during say early embryonic development takes place by vasculogenesis. As it is now known that under certain conditions endothelial precursor cells present in the bone marrow may be recruited and contribute to adult angiogenesis, there seems to be an overlap between angiogenesis and vasculogenesis [87–91]. The concept of collateral artery growth had been modified by the introduction of the term arteriogenesis, which describes the growth of arterioles into mature arteries [92]. In other words, arteriogenesis describes the remodeling of pre-existing arterio-arteriolar anastomoses to completely developed and functional arteries [93]. Stimulation of collateral artery growth provides a potential alternative option for the treatment of patients suffering from occlusive arterial disease. By definition, arteriogenesis represents one aspect of angiogenesis. The lymphatic vasculature forms a vessel network that drains interstitial fluid from tissues and returns it to the blood. Lymphatic vessels are an essential part of the body's immune defense. Lymphangiogenesis refers to the formation of lymphatic vessels from pre-existing lymphatic vessels, in a method believed to be similar to blood vessel development or angiogenesis. Lymphangiogenesis is of outstanding significance in lymphatic-associated disorders such as wound healing, lymphedema and tumor metastasis. Lymphatic endothelial cells and extracellular matrix microenvironment represent primary players in lymphangiogenesis [94].

A complete and transient inflammatory response to injury is a prerequisite for successful vascularization of the wound tissue. Inflammatory cells, namely monocytes/macrophages, T lymphocytes and neutrophils, represent an integral component of the angiogenic process. In addition to being rich in cytokines/growth factors, the inflammation site is very rich in oxidants. Among all biological fluids, the highest level of H_2O_2 is found in the wound fluid. Using the Hunt–Schilling cylinder approach to harvest fluid from the site of inflammation, the presence of 0.1–0.3 mM H_2O_2 has been reported in mice with highly efficient healing mechanisms [31]. Current findings indicate that the oxidant-factor in inflammation plays a central role in supporting tissue vascularization [95]. Decomposition of endogenous H_2O_2 at the wound site by adenoviral catalase gene transfer impairs wound tissue vascularization [31]. Consistently, impairment in healing responses is noted in NADPH oxidase deficient mice and humans [22,31,96]. Although thioredoxin peroxidases or peroxiredoxins are well known for their peroxide decomposing function, peroxiredoxin 6 has low affinity to detoxify H_2O_2 and is primarily directed at detoxifying lipid peroxides [97–100]. The observation that overexpression of peroxiredoxin 6 benefits wound closure [101] indicates the need to check lipid peroxidation in a highly oxidizing wound environment. In this context it is important to note that peroxiredoxin possesses function beyond its antioxidant role [102] and that such properties could influence dermal wound closure. Taken together, the current literature supports that while H_2O_2 is useful to facilitate healing, control of oxidative tissue lipid damage is helpful.

4.1. Antioxidants limit angiogenesis

Evidence demonstrating the ability of antioxidants to oppose vascularization suggests a pro-angiogenic role for oxidants. Antioxidants stall physiological angiogenesis *in vivo* [103,104]. GSH resists tumor angiogenesis [105]. Among thiol antioxidants, pyrrolidine dithiocarbamate (PDTC) inhibits inducible NF-κB, a mediator of inflammation, and arrests myocardial angiogenesis [106]. NF-κB activation is known to be sensitive to a wide range of inducers including H_2O_2 [12,107]. *N*-acetyl-L-cysteine (NAC), an analogue and precursor of GSH, also inhibits angiogenesis by suppressing inducible VEGF gene expression. The thiol antioxidant thiram-tetramethylthiuram disulphide, a chelator of heavy metals, possess anti-angiogenic properties [108]. In cancer biology an anti-angiogenic action of the dietary antioxidant Se, especially methylated Se metabolites, has been recognized as well [109]. Dietary antioxidant flavonoids and polyphenols are known to have potent anti-angiogenic functions in the setting of tumor biology [110–113]. Inducible VEGF release is prevented by flavonoid and phenolic antioxidants [114]. Edible berries, rich in antioxidants, are potently anti-angiogenic both *in vitro* and *in vivo* [115,116]. Quercetin, one of the most abundant flavonoids in edible berries and in the human diet, is a known antioxidant and inhibitor of angiogenesis [117]. The presence of different numbers of phenolic moieties on the B-ring of the flavonols seems to contribute to their antioxidant activity as well as to their potency for resisting angiogenesis [118]. Tea polyphenols are anti-angiogenic as well [119,120]. Resveratrol, a polyphenolic compound found in grapes and other fruits, inhibits angiogenesis [121,122]. The inhibition of VEGF-induced angiogenesis by resveratrol was mediated by disruption of ROS-dependent Src kinase activation and the subsequent vascular endothelial cadherin tyrosine phosphorylation [121]. Polyphenol curcumin (diferuloylmethane, 1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione), obtained from the spice turmeric, exhibits anti-angiogenic activity [123,124]. The soy flavonoid genistein, 4′,5,7-trihydroxyisoflavone, is potently anti-angiogenic and therefore chemopreventive [125,126]. Found in fruits, vegetables, and whole grains commonly consumed by humans, phytoestrogens are antioxidants that include isoflavones, coumestans, and lignans. Anti-angiogenic functions of phytoestrogens have been reported [127]. Known for its ability to prevent the oxidative modification of LDL, probucol is anti-angiogenic [128]. Long-term alpha-tocopherol supplementation is associated with lower serum vascular endothelial growth factor levels [129]. Vitamin C has also been identified as an angiostatic factor [130]. Inducible VEGF and VEGFR-2 expression in vasculature of apolipoprotein-E-deficient mice is down-regulated by vitamins C and E, at least partially through their antioxidant properties [131]. Taken together, a large number of structurally unrelated antioxidants demonstrate angiostatic function suggesting a pro-angiogenic role of endogenous oxidants.

4.2. Oxidants induce VEGF

In biological systems proteins are at risk of oxidative modification and inactivation [132–134]. However, VEGF is protected from oxidative damage by the extracellular chaperone glypican-1 expressed in the vascular system. Glypican-1 can restore the receptor binding ability of VEGF165, which has been damaged by oxidation [135]. Glypican-1 is a member of a family of glycosylphosphatidylinositol anchored cell surface heparan sulfate proteoglycans implicated in the control of cellular growth and differentiation. Among the glypicans, glypican-1 is the only member that is expressed in the vascular system. Glypican-1 interacts with VEGF165 but not with VEGF121. The binding of glypican-1 to VEGF165 is mediated by the heparan sulfate chains of glypican-1, as heparinase treatment abolishes this interaction. Only an excess of heparin or heparan sulfates but not other types of glycosaminoglycans inhibited this interaction. Glypican-1 potentiates the binding of VEGF165 to a soluble extracellular domain of VEGFR2 [135]. It seems that nature has a way of defending VEGF against oxidants because oxidants are required for tissue vascularization [31,136,137]. Recently it has been identified that ROS support electrical field-induced angiogenesis of embryonic stem cells [138]. Today, numerous lines of evidence point towards the conclusion that oxidants support VEGF-dependent tissue function. This section focuses on the induction of members of the VEGF family by oxidants.

At micromolar concentrations, H_2O_2 induces VEGF-A (VEGF165 and VEGF121) expression [139]. Under conditions of co-existence characteristic of any inflammatory site, the effects of TNFα and H_2O_2 on VEGF induction were additive. Using deletion mutant constructs of a 2.6 kb VEGF promoter fragment (bp −2361 to +298, relative to transcription start site) ligated to a luciferase reporter gene it has been established that the sequence from bp −194 to −50 of the VEGF promoter is responsible for the H_2O_2 response. The 144-bp VEGF element (bp −194 to −51) that conferred H_2O_2-mediated transcriptional induction is GC rich and contains four closely-spaced GC boxes (bp −94 to −51) that have been identified to have Sp1 binding function. Studies with Sp1 luciferase reporter constructs have identified that H_2O_2-induced VEGF expression is Sp1 dependent. These studies have also proven that H_2O_2-induced VEGF expression is HIF-independent [139]. The above-mentioned findings have been verified and extended in subsequent studies. Signaling studies identified a cascade comprising Ras-Raf-MEK1-ERK1/2 as the main pathway mediating H_2O_2-induced VEGF-A transcription [140]. In skeletal myotubes, oxidants seem to induce VEGF release via a PI3K/Akt-dependent pathway [141]. Angiotensin II stimulation of VEGF mRNA translation requires production of ROS [142]. Furthermore, angiopoietin 1-induced H_2O_2 plays an important role in Ang1-induced angiogenesis by modulating p44/42 MAPK activity [143]. Several lines of evidence consistent support that mild oxidizing conditions favor VEGF release. Partial cellular glutathione deficiency results in increased VEGF-A release [144]. H_2O_2 modulates vascular permeability via up-regulation of VEGF expression [145].

Evidence supporting the synergism of oxidative stressors in inducing VEGF expression is also present [146]. Studies aiming at characterizing the chemical nature of oxidants capable of inducing VEGF expression provide evidence that H_2O_2, not

hydroxyl radicals, triggers induction of the growth factor. These studies demonstrated that inducible VEGF expression is sensitive to copper and that the angiogenic potential of copper may be harnessed to accelerate dermal wound contraction and closure. Copper shared some of the pathways utilized by hypoxia to regulate VEGF expression [147]. In addition to inducing VEGF transcription, oxidants also enhance VEGF release by increasing VEGF mRNA stability [148]. While oxidant-induced VEGF expression is helpful in the case of dermal wound healing [31], the situation can be different in other disease settings such as H_2O_2 inhalation [145].

Recent studies support the previous observation identifying H_2O_2 as a VEGF-inducing signal and show that H_2O_2 also induces the expression by VEGFR2 by a NF-κB dependent pathway. VEGFR1 was not H_2O_2-sensitive [149]. Oxidized low density lipoprotein, however, down-regulates VEGFR1 minimizing VEGF entrapment by this receptor and improving the availability of VEGF to support angiogenesis [150]. In sum, these findings support that H_2O_2 favors a vascularization response. Mitochondria have been identified as a proximal target specific to H_2O_2-induced signaling and VEGFR2 transactivation [151].

4.3. VEGF signaling: a central role of oxidants

The involvement of oxidants in the VEGF signaling pathway is not limited to induction of VEGF. After VEGF binds to its specific receptors, especially VEGFR2, oxidants seem to be required for the signaling leading to the angiogenic response of VEGF [137,152–154]. ROS are involved in the mitogenic cascade initiated by the tyrosine kinase receptors of several growth factor peptides including VEGF. Insulin induces VEGF expression through H_2O_2 production [155]. Evidence supporting the involvement of ROS in vanadate and hyperoxia-induced expression of VEGF is also reported [156,157]. Although it has been known for a long time that cytokines induce superoxide generation by endothelial cells [158], the physiological significance of such oxidant production remains to be appreciated in full [159]. Early evidence indicating that the binding of VEGF to VEGFR in endothelial cells leads to NADPH oxidase induced oxidant production led to questions about the significance of such oxidants in VEGF signaling [160]. It was soon recognized that VEGF-induced oxidant production was required to activate NF-κB which in turn was required for vascular smooth muscle cell migration, an integral component of angiogenesis [161]. In porcine aortic endothelial cells stably expressing human VEGFR2, receptor activation by VEGF is followed by a rapid rise in intracellular H_2O_2. Genetic and pharmacological studies suggest that such oxidant burst requires as upstream events the activation of phosphatidylinositol 3-kinase and the small GTPase Rac-1 and is likely initiated by lipoxygenases. Inhibition of VEGFR2-dependent generation of ROS attenuates early signaling events including receptor autophosphorylation and binding to a phospholipase *C*-gamma-glutathione *S*-transferase fusion protein. Moreover, catalase, the lipoxygenase inhibitor nordihydroguaiaretic acid, the synthetic ROS scavenger EUK-134, and phosphatidylinositol 3-kinase inhibitor wortmannin all diminish ERK phosphorylation in response to VEGF. Finally, cell culture and stimulation in a nearly anoxic environment mimics the effect of ROS scavenger on receptor and ERK phosphorylation, reinforcing the idea that oxidants are necessary components of the mitogenic signaling cascade initiated by VEGFR2 [162]. EPR evidence also supports that VEGF stimulates superoxide production, which is inhibited by the NADPH oxidase inhibitor, diphenylene iodonium, as well as by overexpression of dominant-negative Rac1 (N17Rac1) and transfection of gp91(phox) antisense oligonucleotides in human umbilical vein endothelial cells [163]. Antioxidants, including *N*-acetylcysteine, various NADPH oxidase inhibitors, and N17Rac1 significantly attenuate not only VEGF-induced VEGFR2 tyrosine phosphorylation but also proliferation and migration of endothelial cells. Importantly, these effects of VEGF are clearly inhibited in cells transfected with gp91(phox) antisense oligonucleotides. In contrast, ROS are not involved in mediating these effects of sphingosine 1-phosphate on endothelial cells [163]. Thus, VEGF-induced endothelial cell signaling and angiogenesis is tightly controlled by the redox microenvironment of the VEGF receptor. Also, NADPH oxidase emerged as a potential therapeutic target for angiogenesis-dependent diseases [163]. IQGAP1 is a scaffolding protein that regulates endothelial cell motility and morphogenesis by interacting directly with cytoskeletal, cell adhesion, and small G proteins, including Rac1. IQGAP1 functions as a VEGFR2-associated scaffold protein to organize ROS-dependent VEGF signaling, thereby promoting endothelial cell migration and proliferation, key components of angiogenesis [164]. The family of proteins involved in redox signaling in response to VEGF–VEGR2 ligation is rapidly expanding. ARF6 is a small GTPase protein involved in membrane trafficking and cell motility. Recently it has been demonstrated that ARF6 is involved in the temporal–spatial organization of caveolae/lipid rafts- and ROS-dependent VEGF signaling in endothelial cells as well as in angiogenesis *in vivo* [165]. It is now established that gp91phox-derived ROS play an important role in mediating VEGF-dependent neovascularization *in vivo* [166].

Hypoxia/reoxygenation-induced changes in endothelial permeability result from coordinated actions of the Rho GTPases Rac1 and RhoA. Rac1 and RhoA rapidly respond to changes in oxygen tension, and their activity depends on NADPH oxidase- and PI3 kinase-dependent production of ROS. Rac1 acts upstream of RhoA, and its transient inhibition by acute hypoxia leads to activation of RhoA followed by stress fiber formation, dispersion of adherens junctions, and increased endothelial permeability. Reoxygenation strongly activates Rac1 and restores cortical localization of F-actin and VE-cadherin. This effect is a result of Rac1-mediated inhibition of RhoA and can be prevented by activators of RhoA, L63RhoA, and lysophosphatidic acid. Cdc42 activation follows the RhoA pattern of activation but has no effect on actin remodeling, junctional integrity, or endothelial permeability. Thus, Rho GTPases act as mediators coupling the cellular redox state to endothelial function [167]. Some of the cytoprotective functions of VEGF are dependent on its ability to induce the mitochondrial antioxidant MnSOD. Receptor ligation of VEGF is uniquely coupled to

MnSOD expression through growth factor-specific ROS-sensitive positive (protein kinase C-NF-κB) and negative (PI3K-Akt-forkhead) signaling pathways [168]. At low concentrations, intra-endothelial H_2O_2 stimulates proliferation or enhances survivals. Also, low concentrations of H_2O_2 stimulate endothelial migration as well as tube formation in an *in vitro* model of angiogenesis. Although low concentrations of H_2O_2 have been shown to be involved in numerous signal transduction pathways and to independently stimulate mitogenesis, there has been little information presented on precisely how mammalian cells respond biochemically to these low concentrations of H_2O_2. Functional proteomic approaches have been employed to identify proteins responsive to low concentrations of H_2O_2 in human endothelial cells [159].

Placental growth factor (PlGF), a member of the VEGF family, acts in synergism with VEGF and eNOS to induce neovascularization [169]. This conclusion was drawn from a study looking at the phenotype of $PlGF^{-/-}$, $eNOS^{-/-}$, $PlGF^{-/-}$ $eNOS^{-/-}$, and wild-type C57BL/6J mice in response to surgically induced hind-limb ischemia. $PlGF^{-/-}eNOS^{-/-}$ double knock-out mice showed the most severe phenotype, including self-amputation and death in up to 47% of the animals studied. In the ischemic legs, capillary density was severely reduced. Macrophage infiltration and oxidative stress were increased as compared to the other groups of animals. These changes were associated with an up-regulation of both iNOS and VEGF in the ischemic limbs [169]. While it is clear that NADPH oxidase-derived H_2O_2 is a key player in VEGF signaling, little is known about the redox control of PlGF signaling. Early placental development is characterized by rapid cell differentiation and migration, matrix remodeling and angiogenesis. NADPH oxidase activity has been studied in placental tissues in early pregnancy and at term. In human placentas from normal deliveries at term substantial basal NADPH activity has been detected. The activity was almost threefold higher in early pregnancy [170]. Whether such increase is implicated in tissue vascularization during early placental angiogenesis remains to be determined.

In addition to members of the VEGF family, other factors that support wound angiogenesis are subject to redox control. Fibroblast growth factor 2 (FGF2) stimulates endothelial migration [171]. Delivery of FGF genes to wound repair cells enhances arteriogenesis [172]. Hypoxia serves as a physiologic cue to drive an angiogenic response via HIF-dependent mechanisms. ROS are known to support hypoxia signaling [173–176]. Minor elevation of lactate levels in the tissue produces the angiogenic effects of hypoxia under aerobic conditions. Recent evidence demonstrates that lactate, accumulated at the wound site in high mM concentrations, stimulate wound angiogenesis via a redox-sensitive mechanism [177].

5. Nitric oxide in wound healing

In the late 1970s, research was unfolding that implicated nitric oxide involvement in the process of vasodilation. By 1986, research culminated in the identification of nitric oxide as the endothelium-derived relaxing factor responsible for the maintenance of vascular tone, thus implicating nitric oxide as a potential wound-healing agent. Numerous aspects of redox biology are sensitive to nitric oxide (NO), a nitrogen-centered free radical gas. For example, both superoxide and nitric oxide are ubiquitous free radicals which when co-exist rapidly react to produce deleterious peroxynitrite. On their own, however, both superoxide-derived H_2O_2 as well as NO are critically important in driving numerous processes that are central to wound healing. Superoxide anion and NO function separately and interactively as cellular second messengers [11,178]. For example, superoxide anion and NO play an intrinsic role in the regulated ordered turnover of proteins, rather than randomly cause protein damage and their inactivation. The vasorelaxant functions of NO counteracted by superoxide which not only traps NO [179] but through the resulting peroxynitrite blocks prostacyclin synthase by nitration of an active site tyrosine residue [180]. The possible implications of H_2O_2 in wound healing have been critically discussed above. This section is aimed at critically highlighting the potential effects of NO on dermal wound healing. Detailed review of the role of NO on wound healing is presented elsewhere [181–184].

Ischemic wounds signal for hypoxia by HIF-dependent mechanisms. A functional HIF response requires stabilization of the alpha-subunit, *e.g.* HIF-1alpha, during hypoxia and dimerization with HIF-1beta, to drive target gene activation [185]. Intriguingly, high concentrations of NO stabilize HIF-1alpha and thus mimic a hypoxic response under normoxia. However, during hypoxia low concentrations of NO facilitate destruction of HIF-1alpha and thus reverse HIF signaling [186]. The significance of NO in oxygen sensing in the ischemic wound remains to be elucidated.

Over a decade ago early reports from the laboratory of Adrian Barbul presented pointed evidence establishing the significance of NO in cutaneous wound healing [187]. Maximal NO synthase activity is noted early in cutaneous wound healing, with sustained production up to 10 days after wounding. Wound macrophages represent a major source of nitric oxide production in the early phase of wound healing [188]. Inhibition of wound NO synthesis lowered wound collagen accumulation and wound breaking strength suggesting that NO synthesis is critical to wound collagen accumulation and acquisition of mechanical strength. Later it was demonstrated that wound fibroblasts are phenotypically altered during the healing process to synthesize NO, which, in turn, regulates their collagen synthetic and contractile activities [189]. The blockade of NO synthesis impairs cutaneous wound healing, acting in early and late phases of wound repair [190].

Interestingly, impaired diabetic wound healing is associated with decreased wound NO synthesis [191]. Cutaneous wound healing is associated with the expression of genes encoding proteins that synthesize NO [192,193]. While excessive NO at the wound site can be clearly toxic [194,195], evidence supporting the favorable biological significance of endogenous NO is compelling. Triggering of VEGF expression is a crucial molecular mechanism underlying NO function during wound healing [196]. NO regulates other wound-associated cytokines and chemotactic factors as well [197]. Furthermore, the presence of a functionally active iNOS is a crucial prerequisite for normal wound re-epithelialization [198]. Of note, although generation

of NO by iNOS has been shown to be required for cutaneous wound healing, no differences have been noted in incisional healing between iNOS knock-out and wild-type mice. eNOS, however, plays a significant role in facilitating wound repair and growth factor-stimulated angiogenesis [199]. Supplemental dietary arginine, a substrate for NO synthesis, enhances wound healing in normal mice. The loss of a functional iNOS gene abrogates the beneficial effect of arginine in wound healing. This suggests that the metabolism of arginine via the NO pathway is one mechanism by which arginine enhances wound healing [200]. In diabetics, impaired NO synthesis at the wound site can at least partially be reversed by arginine supplementation [201]. Exogenous NO released from a hydrogel wound dressing has potential to benefit wound healing [202,203].

6. Conclusion

In sum, numerous aspects of wound healing are subject to redox control. Thus, development of a thorough understanding of how endogenous ROS generated in wound-related cells may influence the healing process becomes critically important. Such an understanding could result in novel redox-based strategies to treat wounds. Current results with growth factor therapy of wounds do not meet expectations. Many of these growth factors, like PDGF, rely on ROS for functioning. Thus, redox-based strategies may serve as effective adjuncts to jump-start healing of a chronic wound. With hypoxia being a characteristic feature of most problem wounds, it is reasonable to assume that correction of wound pO_2 may facilitate generation of endogenous ROS by NADPH oxidases in wound-related phagocytic and non-phagocytic cells. Therapeutic modalities relying on up-regulating ROS generation in the wound microenvironment will have to be dealt with caution. While a window of opportunity seems to exist under conditions of low concentrations of ROS, high levels of ROS clearly have the potential to complicate regeneration and remodeling of nascent tissue.

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