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Redox Signaling in Diabetic Wound Healing Regulates Extracellular Matrix Deposition

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Abstract

Significance: Impaired wound healing is a major complication of diabetes, and can lead to development of chronic foot ulcers in a significant number of patients. Despite the danger posed by poor healing, very few specific therapies exist, leaving patients at risk of hospitalization, amputation, and further decline in overall health.
Recent Advances: Redox signaling is a key regulator of wound healing, especially through its influence on the extracellular matrix (ECM). Normal redox signaling is disrupted in diabetes leading to several pathological mechanisms that alter the balance between reactive oxygen species (ROS) generation and scavenging. Importantly, pathological oxidative stress can alter ECM structure and function.
Critical Issues: There is limited understanding of the specific role of altered redox signaling in the diabetic wound, although there is evidence that ROS are involved in the underlying pathology.
Future Directions: Preclinical studies of antioxidant-based therapies for diabetic wound healing have yielded promising results. Redox-based therapeutics constitute a novel approach for the treatment of wounds in diabetes patients that deserve further investigation. *Antioxid. Redox Signal.* 27, 823–838.

Keywords: diabetes, wound healing, reactive oxygen species, extracellular matrix, collagen

Introduction

Diabetes is widespread in the United States, and its complications have devastating effects on health and quality of life (1, 2, 174). One of the most serious complications of diabetes is impaired wound healing, which leads to the development of chronic wounds in the lower extremities in 15–25% of diabetes patients (27, 28, 30, 167, 194). Chronic wounds significantly decrease mobility, social functioning, and overall health, and are the leading cause of hospitalization and limb amputation in diabetes patients (28, 144, 167, 174). In addition, management of diabetic wounds is a major economic burden, generating $13 billion in healthcare costs per year in the United States (28, 194). Conventional wound care practices can be effective in diabetes patients, but a large fraction of diabetic ulcers still persist (10–15%) or lead to amputation (5–24%) 6–18 months after diagnosis (8, 27, 119, 128). Novel therapeutic strategies must focus on the pathological mechanisms underlying impaired healing in diabetes to improve patient outcomes.

Aberrant redox signaling and increased oxidative stress are widely accepted contributors to the development of diabetic complications, including cardiovascular disease, nephropathy, and retinopathy (12, 33, 68, 112, 145, 161). Oxidative stress also plays a significant role in regulating normal wound healing by facilitating hemostasis, inflammation, wound closure, and development and maturation of the extracellular matrix (ECM) (53, 149, 152–154, 156). The ECM is an important mediator of healing—it provides structure, coordinates cell–matrix and cell–cell interactions, and facilitates signal transduction in the wound. This review will examine the role of oxidative stress in the etiology of impaired healing in diabetes, with a particular focus on the ECM, and discuss the development of treatment strategies based on these principles.

Wound Healing in Diabetes

Dermal wound healing [reviewed extensively in Refs. (19, 72, 160, 185)] is a highly coordinated process that occurs in

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three overlapping phases: (i) inflammation, which includes hemostasis, inflammatory cell recruitment, and cytokine and growth factor secretion; (ii) proliferation, which is characterized by formation of the provisional matrix, angiogenesis, and re-epithelialization; and (iii) remodeling, in which granulation tissue is reorganized and the mature scar is formed (Fig. 1). In the diabetic wound, each of these phases is compromised, disrupting and delaying the orderly progression of healing (Fig. 2) (26, 59, 167). The bulk of controlled studies involving diabetic wounds have been performed in animal models. It is widely accepted that these do not fully recapitulate the human disease, so researchers often employ multiple animal models to study wound healing (91). This review is largely based on studies of diabetic wound healing in animals, coupled with data from controlled human studies when available.

The inflammation phase in diabetic wound healing is prolonged but ineffective (3, 163, 167, 183). Diabetes is characterized by chronic systemic inflammation, evidenced by increased baseline expression of inflammatory markers, including macrophage chemoattractant protein-1, tumor necrosis factor (TNF), interleukin-6 (IL-6), and soluble P- and E-selectins, in blood collected from diabetes patients (87, 125, 181). This pattern of expression of proinflammatory factors influences inflammation in response to injury, and is characteristic of chronic ulcers in a variety of settings (52, 195). After injury in diabetes, neutrophils and macrophages are slowly recruited to the wound, but remain in the wound bed in large numbers for an extended period of time (3, 65, 106, 182, 183). This creates an environment that is particularly enriched in proinflammatory cytokines (such as IL-1β, IL-6, and TNF-α) and reactive oxygen species (ROS), which further damage the tissue and stall proliferation of fibroblasts and keratinocytes essential for the later phases of healing (3, 167, 183). Macrophages in diabetic wounds also exhibit reduced phagocytic capacity, which allows bacteria and debris to accumulate and decreases expression of growth factors, such as vascular endothelial growth factor (VEGF) (26, 59, 88, 167). These defects limit angiogenesis and progression to the proliferation phase (3, 167, 183).

In diabetic wounds, the proliferation phase is characterized by impaired granulation tissue formation. Granulation tissue comprises an ECM produced by fibroblasts and new blood vessels formed by invading endothelial cells and serves as a scaffold for keratinocyte migration and wound closure. Decreased expression of growth factors (such as VEGF and TGF-β) diminishes the proliferation, migration, and differentiation of fibroblasts, endothelial cells, and keratinocytes (26, 78). Diabetic wound fibroblasts also have abnormal morphology, decreased adhesion, diminished response to growth factors and cytokines, and decreased production of collagens and fibronectin (FN) (9, 71, 94, 189). This results in abnormal ECM structure and composition (Fig. 3) (163). Moreover, the ECM is damaged by overexpression of matrix metalloproteinases (MMPs) and decreased expression of their inhibitors (tissue inhibitors of metalloproteinases [TIMPs]) (26, 29, 102, 103). The MMP/TIMP imbalance also leads to growth factor degradation, which interrupts signaling vital to endothelial cell and keratinocyte migration, and therefore impairs angiogenesis and re-epithelialization (15, 26, 37, 59, 81).

The remodeling phase is similarly impaired by the proteolytic environment in diabetic wounds. Excessive breakdown of ECM proteins and the formation of abnormal protein–protein bonds disrupt normal formation of the mature collagen matrix and permanent scar (11, 26, 117, 167). This can lead to decreased scar thickness (depth), as is observed in the type 2 diabetic Zucker rat and other animal models; such

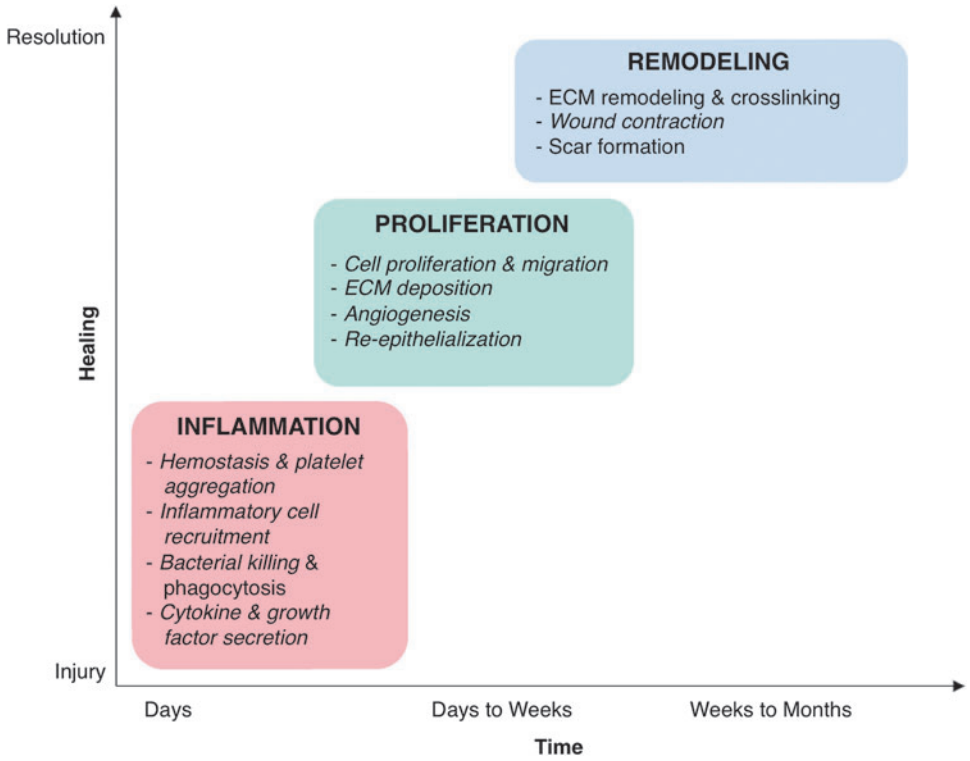

FIG. 1. Redox control of dermal wound healing. Normal wound healing occurs in three overlapping phases: inflammation, proliferation, and remodeling. Progression through these phases is highly regulated and coordinated by several mechanisms, including redox signaling. Both generation and scavenging of ROS, particularly H_2O_2, are critical to normal healing. The major processes regulated by redox signaling in each phase of healing are indicated in italics. ECM, extracellular matrix; H_2O_2, hydrogen peroxide; ROS, reactive oxygen species. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

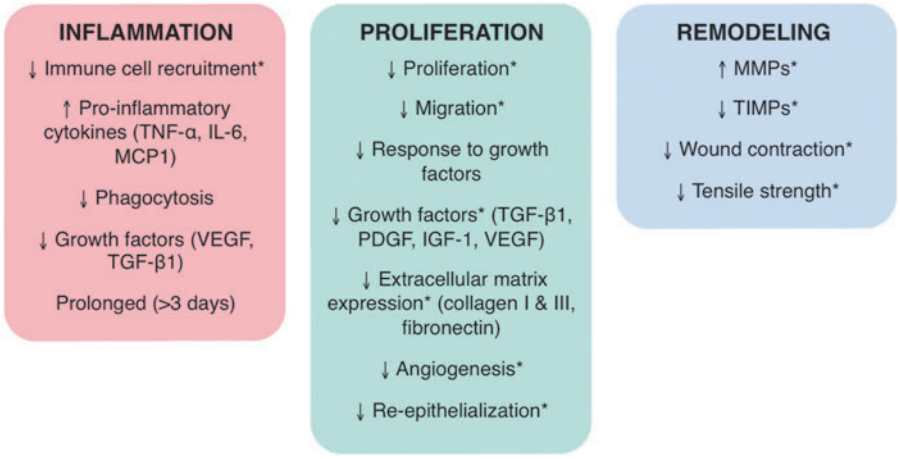

FIG. 2. Wound healing in diabetes. In contrast to normal healing, wound healing in diabetes is uncoordinated and spatiotemporally disorganized. Chronic diabetic wounds do not progress smoothly through inflammation, proliferation, and remodeling; they are instead characterized by an extended inflammation phase, a limited proliferation phase, and irregular remodeling. The critical changes in each phase of healing in diabetes are identified. Healing processes that involve ECM, a critical facilitator of healing because of its role as structural support and a mediator of cellular interactions, are indicated by *asterisk* (*). IGF-1, insulin-like growth factor-1; IL-6, interleukin-6; MCP-1, macrophage chemoattractant protein-1; MMP, matrix metalloproteinase; PDGF, platelet-derived growth factor; TGF-β1, transforming growth factor-β1; TIMP, tissue inhibitors of metalloproteinase; TNF-α, tumor necrosis factor-α; VEGF, vascular endothelial growth factor. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

changes in ECM deposition in the scar may reduce tensile strength and make the skin more susceptible to damage and reinjury (117, 150, 163). Decreased wound contraction during remodeling also contributes to reinjury risk; in the Zucker rat, a greater portion of healed skin is composed of scar tissue, which is significantly weaker than normal skin (163). Chronic wound development in diabetes is influenced by myriad defects in signaling, cell function, and ECM structure throughout the healing process.

ECM in Diabetic Wound Healing

ECM is a critical facilitator of wound healing, from its beginnings as a fibrin clot through remodeling into granulation tissue and a permanent scar [reviewed in Refs. (4, 64, 139, 170)]. Wound ECM not only provides structure and support to the tissue, but also serves as a reservoir for growth factors and mediates cell–cell, cell–matrix, and matrix–protein interactions. Through these interactions, ECM influences cell behavior and function (including adhesion, proliferation, migration, differentiation, and gene expression), and thus its own remodeling and maturation. The importance of ECM is reflected in the diabetic wound, where its irregularity has numerous effects on wound fibroblasts, keratinocytes, and endothelial cells, as already described. In diabetes, the structure and function of ECM is marred by fibroblast dysfunction, changes in protein deposition, degradation, and remodeling, and post-translational modification by advanced glycation end products (Fig. 4).

Fibroblast function in diabetes

Fibroblasts are key to ECM production and remodeling in wound healing, but several of their essential functions are compromised in diabetes. For example, fibroblasts isolated from diabetic wounds proliferate more slowly than fibroblasts isolated from uninjured skin and nondiabetic chronic wounds (79). Diabetes also induces fibroblast apoptosis; there are increased TUNEL and caspase-3 positive fibroblasts in diabetic gingival wounds than in normal controls (49). Similarly, hyperglycemia has been shown to inhibit proliferation and induce apoptosis in dermal fibroblasts *in vitro* (78, 189). Migration is similarly reduced; fibroblasts derived

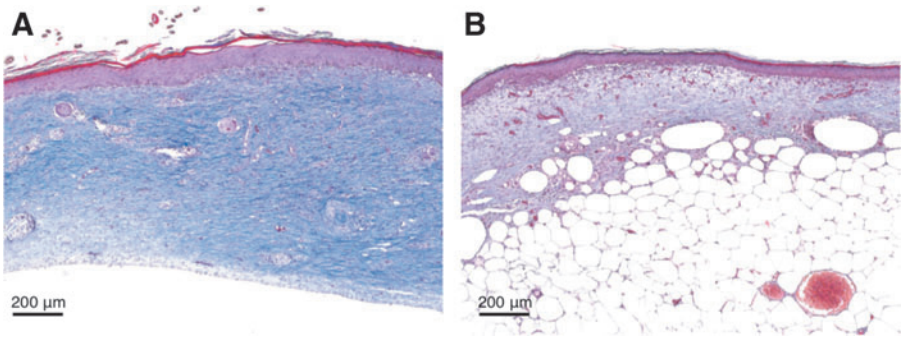

FIG. 3. ECM deposition is reduced in diabetes. Reduced deposition of ECM is characteristic of wound healing in diabetes. Masson's trichrome staining of mouse granulation tissue of healthy C57BL/6 mice **(A)** and diabetic db/db mice **(B)** reveals significantly reduced collagen deposition and maturation (*blue*). Wounds were explanted 14 days postinjury.

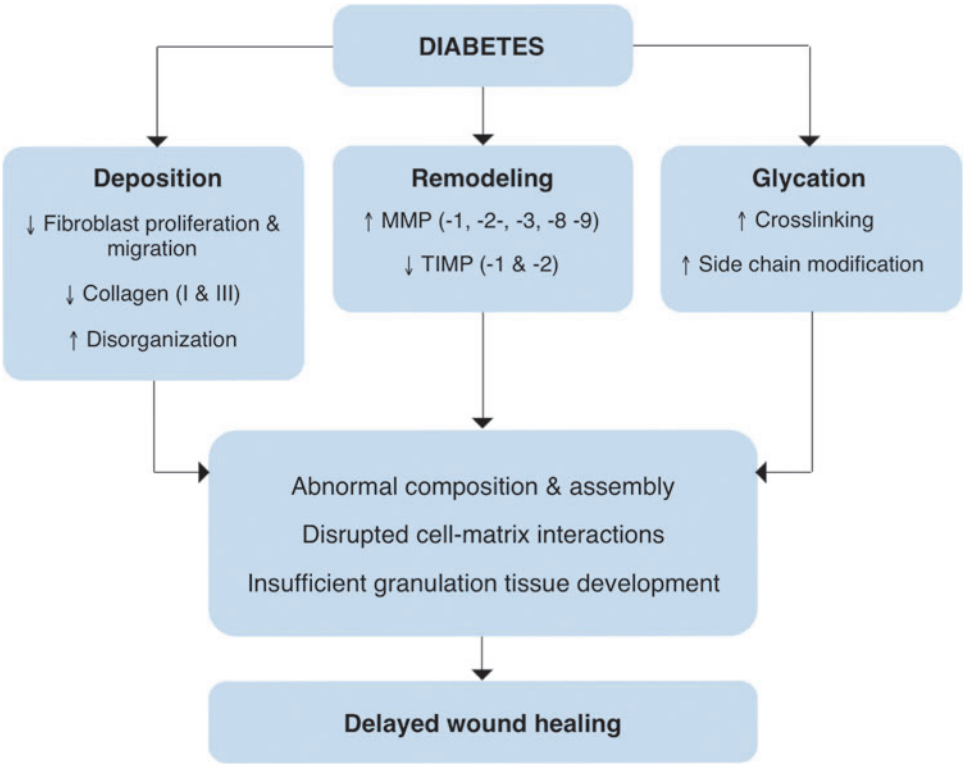

FIG. 4. Changes in ECM in diabetes. The structure and function of the ECM are altered in diabetes *via* changes in fibroblast function, post-translational modification by glucose (glycation), and an imbalance of ECM deposition and remodeling. These changes influence matrix composition and assembly, cell–matrix interactions, and development of granulation tissue, and ultimately contribute to delayed wound healing in diabetes. Changes described in this figure have been found in human diabetic tissues and wounds. TIMP, tissue inhibitors of metalloproteinase. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

from the db/db mouse exhibit decreased invasion in Boyden chamber assays, and high glucose impairs migration of normal fibroblasts by suppressing c-Jun n-terminal kinase (JNK) phosphorylation *in vitro* (97, 189). Moreover, fibroblasts isolated from diabetic wounds respond abnormally to TGF-β1, a growth factor that induces collagen and ECM synthesis, and retain collagen intracellularly (110). All together, these defects in fibroblast function result in poor ECM deposition, which is discussed in detail below.

ECM deposition in diabetes

The deposition of collagen, the most abundant ECM protein in normal tissue and the healing wound, is significantly altered in diabetes (Fig. 3). At baseline, skin biopsies from diabetes patients exhibit lower expression of collagens I and III, as detected by Western blot and immunohistochemistry (22, 192). The ratio of collagen I to collagen III, which is correlated with ECM tensile strength, is also reduced (22). Moreover, the ECM in diabetic skin is more disorganized, with increased spacing between collagen fibrils (123, 192). Diabetes may also impact collagen fibril diameter, but it remains unclear whether fibril thickness is increased or decreased (123, 192). Similar decreases in collagen I and collagen III levels have been reported in diabetic rodent models, and histological analysis reveals degeneration of collagen fibers and disorganized epithelial structure as shown in human skin (39, 92, 93, 146). Real-time PCR analysis has shown increased *Col1a2*, *Col3a1*, and *Col5a1* gene expression in patients with low skin collagen levels, which suggests post-transcriptional regulation of collagen in diabetes, but most studies report only protein or hydroxyproline content (22, 123, 192). In addition, gene expression levels of collagens I, III, IV, V, VI, XIV, and XVII are decreased in diabetic rats, which is consistent with the collagen protein levels in other diabetic rodent models (146, 163).

The collagen levels in diabetic wounds are also significantly decreased, as determined from hydroxyproline assay and Masson's trichrome staining of diabetic human and mouse wounds (25, 74, 80, 120). Specifically, collagen I and III levels are decreased compared with those in nondiabetic wounds, although *Col1a1* and *Col3a1* gene expression was elevated in diabetic wounds in one study (34, 163). Notably, diabetic wounds exhibit increased expression of miR-29a, a key negative regulator of collagen I and collagen III expression, which is a potential mechanism of post-transcriptional regulation of collagen in diabetes (34, 114). Despite the major deficits in collagen deposition in diabetic wounds, few studies have addressed mechanisms that mediate changes in transcription or post-transcriptional regulation of collagen in this environment, such as decreased TGF-β1 signaling and microRNA regulation (6, 24, 55, 75, 110).

FN is another major component of granulation tissue and an essential antecedent of collagen I deposition. FN is elevated in the dermis of chronic diabetic ulcers analyzed by immunohistochemistry (and persists 12–18 months post-wounding), but has also been reported to be highly fragmented in wounds and diseased gums in diabetes patients (106, 165). FN is overrepresented in ECM produced by diabetic ulcer-derived fibroblasts, but the same fibroblasts exhibit dampened FN expression in response to TGF-β1 stimulation (110). One study reported a threefold decrease in FN RNA expression in diabetic ulcers, but this was in comparison with normal uninjured skin, which complicates interpretation (61). Although further study is required to fully understand FN expression in diabetic wounds, multiple studies have shown that treatment with exogenous FN improves healing rate and hydroxyproline content in diabetic wounds (73, 138).

ECM remodeling in diabetes

MMPs, which cleave collagen, FN, and other components of the ECM, are highly active in diabetes (175). Skin biopsy samples from diabetes patients exhibit increased expression of active MMP-1, MMP-2, and MMP-9, as determined by ELISA and gelatin zymography (95, 192). Similarly, MMP-2 and MMP-3 are elevated in the skin in rat models of diabetes (92, 93). Wound tissue homogenates from diabetes patients have significantly elevated levels of MMP-2, MMP-3, MMP-8, and MMP-9 compared with nondiabetic controls, and analysis of diabetic wound exudate also demonstrates elevated MMP-2 and MMP-9 (102, 177). Moreover, fibroblasts derived from diabetic wounds secrete more MMP-2 and MMP-3 in culture (178). Comparably, MMP-2, MMP-3, and MMP-13 expression is increased in wounds of the diabetic Zucker rat, and MMP-9 activity is increased in granulation tissue of diabetic mice (C57BL/6-db) (146, 163). One study has indicated that MMP-2 and MMP-9 are actually decreased in diabetic mouse wounds, but these conclusions were based on RNA expression data rather than protein quantification (177). Diabetic wounds also typically contain high levels of bacterial proteases, which can activate human proteases, including MMP-2 (116).

Elevated MMP activity in diabetes is compounded by decreased expression of TIMPs, which bind to and inhibit activated MMPs. TIMP-1 and TIMP-2 levels are decreased at baseline in skin biopsy samples from diabetes patients and rodent models (92, 93, 192). TIMP-2 is also reduced in diabetic wound homogenates (102). An increase in MMP/TIMP ratio disrupts the normal balance of ECM synthesis and degradation, which impacts ECM composition and fragmentation. Specifically, high MMP-9/TIMP ratio is predictive of poor healing in diabetes patients (99). Because of this, there is significant interest in targeting MMP activity to treat diabetic wounds; one recent study demonstrated that MMP-9 knockout or inhibition improved healing in diabetic mice (66).

ECM glycation in diabetes

In diabetes, ECM structure and function is also changed by glycation, a nonenzymatic reaction between glucose and proteins (39, 123, 124). Glycation leads to the formation of intermolecular crosslinks, which significantly alter the biomechanical properties of ECM (69). For example, *in vitro* glycation of skin biopsy samples increases direction-dependent stiffness of the tissue (143). Similarly, glycated collagen matrices are less flexible and more rigid than nonglycated collagen matrices, which impairs their contraction by myofibroblasts, an essential aspect of scar formation (100, 134). Glycation of collagen side chains alters the overall charge of the molecule, which interferes with its interaction with other matrix components and disrupts normal matrix assembly (67, 69). Glycated collagen is also more resistant to MMP-mediated degradation, which disrupts matrix remodeling (45, 69).

ECM glycation also alters cell–matrix interactions and cell behavior (14, 134). For example, contact with glycosylated matrix induced cell cycle arrest and apoptosis in cultured human dermal fibroblasts, an effect mediated by activation of the receptor for advanced glycation end products (RAGE) (39, 124). This increase in apoptosis is consistent with the increased TUNEL staining observed in histological sections of human diabetic wounds (123, 124). Furthermore, fibroblasts cultured on glycated collagen exhibit decreased migration because of poor integrin binding and reduced expression of collagen, FN, elastin, and MMP-1 (100, 123, 170). Keratinocytes and endothelial cells similarly exhibit reduced migration and adhesion on glycated ECM (134). Multiple studies have attempted to improve wound healing in diabetes by inhibiting ECM glycation; however, treatment with aminoguanidine, which decreases the formation of advanced glycation end products (AGE), has yielded mixed results, although differences may be related to the diabetes models used (21, 191).

Redox Signaling in Wound Healing

ROS, including superoxide (O_2^-), hydrogen peroxide (H_2O_2), hydroxyl radical, and other reactive oxygen derivatives, are produced in the cell as an unavoidable byproduct of oxidative phosphorylation. ROS can damage cells by oxidizing lipids and proteins, so levels are tightly controlled by the presence of ROS scavenging enzymes and small molecule antioxidants. Despite the potential harm posed by ROS, signaling through these molecules is essential for many cellular processes (154).

Redox signaling regulates several wound healing processes (Fig. 1) [reviewed in Refs. (53, 149, 154, 156)]. H_2O_2, a reactive species produced by dismutation of O_2^-, acts as the principal secondary messenger in wound healing and is present at low concentrations (100–250 μM) in normal wounds (53, 149). The critical role of ROS in healing has been shown in systems with NADPH oxidase (Nox) deficiency or antioxidant overexpression; wounds with low levels of ROS because of these defects exhibit impaired angiogenesis, abnormal remodeling, and delayed closure in patients and mouse models (62, 98, 147).

Redox regulation of wound healing begins in the inflammation phase, when ROS levels peak (130). Platelet aggregation in response to collagen induces production of O_2^- and H_2O_2, which facilitate further aggregation and platelet recruitment (46, 135, 156). O_2^- and H_2O_2 also modulate platelet aggregation in response to various stimuli *in vitro*, including collagen, adenosine diphosphate (ADP), and arachidonic acid (10, 173). ROS generated during hemostasis may also contribute to inflammatory cell recruitment to the wound by stimulating chemotaxis and adhesion molecule expression (105, 154). High levels of O_2^- and H_2O_2 are generated by neutrophils and macrophages *via* Nox, which is rapidly expressed after wounding, and subsequent dismutation (53, 149, 154, 156). This ''oxidative burst'' serves as the primary mechanism of bacterial killing and prevention of wound infection, and is accompanied by a temporary downregulation of some ROS scavenging enzymes (148, 156). ROS also stimulate release of cytokines and growth factors, including macrophage colony-stimulating factor, platelet-derived growth factor (PDGF), and TNF-α.

Redox signaling is also critical for the proliferation phase. ROS promote fibroblast proliferation and migration, and mediate TGF-β1 signaling, which results in migration, collagen and FN production, and basic fibroblast growth factor (bFGF) expression (7, 176, 193). ROS also facilitate angiogenesis; H_2O_2 stimulates VEGF expression by macrophages,

keratinocytes, and fibroblasts independent of hypoxia inducible factor, and is required for signaling downstream of VEGF receptor binding (35, 40, 147, 155, 156). Furthermore, exogenous H_2O_2 induces endothelial cell migration, and low levels of exogenous H_2O_2 increase angiogenesis in mouse wounds (56, 105). H_2O_2 also stimulates keratinocyte proliferation and migration, facilitating re-epithelialization (104).

ROS generated in wounds are tightly regulated by ROS scavenging enzymes, such as superoxide dismutases (Cu/ZnSOD, MnSOD, and SOD3), peroxidases (catalase [CAT], phospholipid hydroperoxide glutathione peroxidase), and peroxiredoxins, as well as small molecule antioxidants, such as vitamin E and glutathione (149, 166). Many of the enzymes are upregulated in healing wounds, whereas levels of the small molecule antioxidants drop as they are depleted by ROS (149). Disrupting the redox balance provided by these antioxidant enzymes is sufficient to make wounds chronic, as does overwhelming the antioxidant mechanisms by adding exogenous H_2O_2 (50, 105, 147). A balance of ROS generation and scavenging is required for efficient and timely wound healing.

Redox Signaling in Diabetes

ROS levels are elevated in various tissues in diabetes patients through a combination of mechanisms that increase ROS production and reduce antioxidant defenses (Fig. 5) (68, 112). Thus, diabetic wounds are characterized by high levels of ROS, particularly O_2^- and H_2O_2 (50, 126, 179, 189). Several pathological mechanisms contribute to the accumulation of ROS in diabetic wounds, all of them secondary to hyperglycemia (33, 57, 68, 133). When considered with the central role of redox signaling in wound repair, the redox imbalance in diabetic wounds described hereunder has major implications for the pathogenesis of delayed healing in diabetes.

Mitochondrial superoxide production

High glucose significantly increases O_2^- levels in cells and skin *in vitro* through the mitochondrial electron transport chain (42, 109, 133, 157). Superoxide is an unavoidable by-product of oxidative phosphorylation, but under normal conditions, <10% of all oxygen consumed in aerobic metabolism is reduced to O_2^- (68, 133). Hyperglycemia increases O_2^- production by increasing the amount of pyruvate oxidation in the TCA cycle and consequently the availability of electron donors NADH and $FADH_2$. Increased electron flux then increases the proton gradient across the inner mitochondrial membrane, which at a critical threshold disrupts electron transport through complex III (68). Then, electron transport is largely mediated by coenzyme Q, which transfers only one electron to oxygen, producing excess O_2^- and H_2O_2 (Fig. 6) (68, 122).

Excessive superoxide production in the mitochondria further impacts ROS levels by altering the flux through several intracellular pathways (33, 67, 68, 122). ROS leads to glyceraldehyde 3-phosphate dehydrogenase (GAPDH) inhibition by poly(ADP-ribose) modification, which increases levels of glycolysis intermediates upstream of GAPDH. This provides increased substrate levels for the polyol, protein kinase C (PKC), and hexosamine pathways (68, 122, 129). Activation and interaction of these pathways ultimately alter gene expression, deplete antioxidant resources, and favor the production of advanced glycation end products (60, 68, 122, 133).

Advanced glycation end products

AGE are formed through nonenzymatic reactions between glucose or other reducing sugars and proteins. The carbonyl group of the sugar reacts with the free amino group of an amino acid, such as lysine or arginine, to form a Schiff base (69). Then, rearrangement leads to the formation of a stable

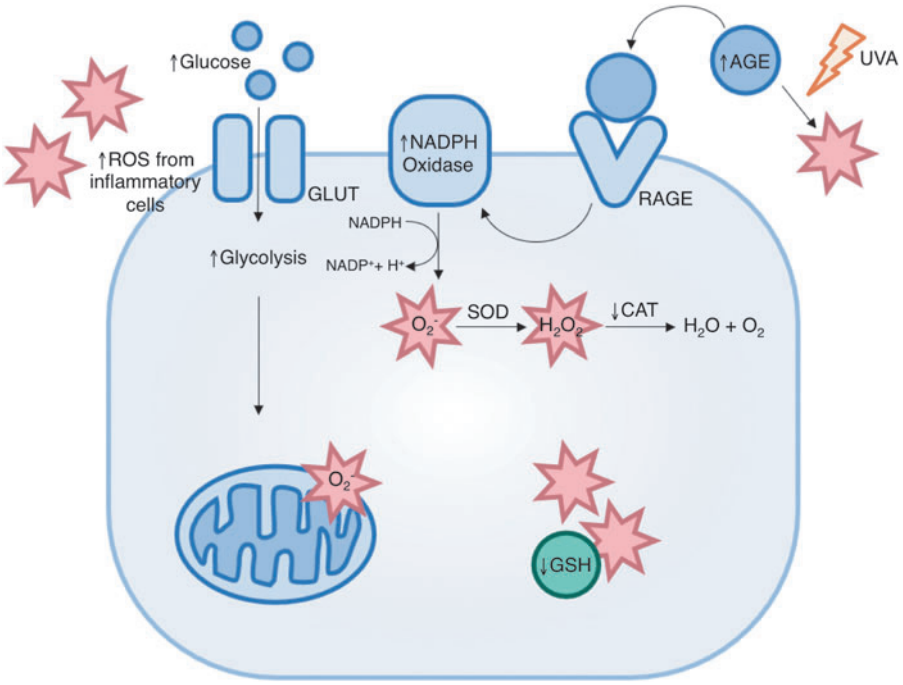

FIG. 5. Sources of oxidative stress in diabetic wounds. Several mechanisms contribute to increased ROS levels in diabetes (indicated by *stars*). These include increased mitochondrial superoxide production, formation of advanced glycation end-products, increased activity of ROS-generating enzymes such as NADPH oxidase, and decreased expression of antioxidant enzymes and small molecules. AGE, advanced glycation end products; CAT, catalase; GLUT, glucose transporter; GSH, glutathione; RAGE, receptor for advanced glycation end products; SOD, superoxide dismutase. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

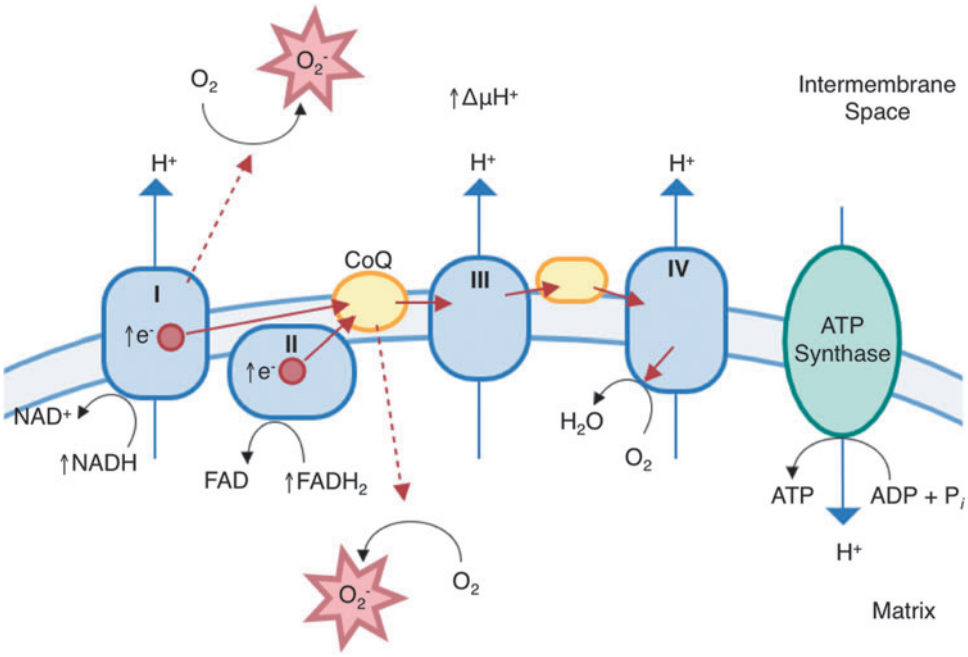

FIG. 6. Excess mitochondrial superoxide production in diabetes. Hyperglycemia induces excess superoxide production by increasing the number of electron donors available to the electron transport chain. This increases the proton gradient past a critical level, and allows electron leakage (indicated by *dashed lines*) at complex I and CoQ. CoQ, coenzyme Q. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

Amadori product, which may be further rearranged, condensed, oxidized, or dehydrated to form new AGE or cross-links and adducts with additional proteins (68, 69). AGE accumulate more rapidly in high glucose cell culture as well as diabetic patients and animal models (67, 127, 159, 188).

AGE increase intracellular ROS levels by several mechanisms, even in normal glucose conditions (38, 42, 115, 159). AGE binding with the RAGE produces O_2^- and H_2O_2 through activation of Nox and by increasing the expression of Nox subunits, including Nox4 and $p22^{phox}$ (38, 107, 159, 163, 188). This ROS production has been shown to exacerbate excessive mitochondrial superoxide production in diabetes; cytosolic H_2O_2 produced after RAGE binding decreases the activity of complex I, resulting in increased superoxide leakage in diabetic conditions (42). ROS produced by the mitochondria, in turn, increase RAGE expression, perpetuating further ROS generation (18). There is also evidence that AGE induce ROS production through $\alpha 1\beta 1$ integrin binding and Nox activation independent of RAGE and under UVA radiation, which is particularly relevant in the skin (107, 131). AGE-induced ROS production in endothelial cells has been inhibited by treatment with an anti-RAGE antibody, but further research must be done to address RAGE-independent mechanisms of ROS generation by AGE (18).

Increased ROS-generating enzymes

ROS production by several ROS-generating enzymes is elevated in diabetic wounds. As already discussed, expression and activity of Nox, the major source of ROS in many cell types, are increased in response to RAGE binding (132). Nox activity is also increased downstream of hyperglycemia-induced PKC activation in smooth muscle and endothelial cells (82). PKC phosphorylates the $p47^{phox}$ subunit of Nox, which induces its translocation to the cell membrane and assembly of the functional Nox complex (133). Similarly, hyperglycemia-induced angiotensin II type 1 receptor (AT1) activation increases expression of $p47^{phox}$ and enhances ROS production by Nox (136). AT1 is expressed by several cell types in the wound, including myofibroblasts and keratinocytes (169). In addition, expression of Rac2, an activator of Nox, is elevated in the Zucker rat model, but the mechanism of its upregulation has not been determined (163).

Expression and activity of H_2O_2-producing enzymes xanthine oxidase (XO) and p66Shc are significantly increased in diabetic mouse wounds, and healing is improved when either protein is knocked down (58, 180). XO and p66Shc are also elevated in fibroblasts cultured in high glucose, but the mechanisms mediating their increased expression remain unknown (58, 180). Deeper understanding of the molecular mechanisms underlying increased activity of ROS-generating enzymes in diabetic wounds is needed.

Decreased ROS-scavenging mechanisms

Increased production of ROS in diabetes is coupled with a reduction in antioxidant defenses, which intensifies the redox imbalance (67, 164, 179). Levels of glutathione, a free radical scavenger, are significantly reduced in wound tissue from diabetic patients and mouse models (13, 121). Nitric oxide (NO), which can neutralize O_2^-, is also reduced in diabetic wounds, but its role in the redox balance of diabetic wounds remains unclear (109, 112).

The expression of antioxidant enzymes is also reduced in diabetes. CAT levels are low in diabetic mice at baseline, and lymphocytes isolated from diabetes patients exhibit decreased CAT activity (13, 141). Analysis of blood collected from diabetes patients showed reduced SOD, CAT, and glutathione peroxidase activity, and an overall decrease in antioxidant status (172). There is conflicting evidence regarding MnSOD expression and activity in diabetes; studies in mice have demonstrated decreased expression and activity in diabetes, whereas analysis of human samples has indicated increased MnSOD activity in diabetic wounds (13, 113, 172). Further characterization of chronic wounds in diabetes must be performed to fully understand the antioxidant activity.

A significant factor that influences antioxidant enzyme levels in diabetes is impaired signaling through the transcription factor nuclear factor erythroid 2-related factor 2 (Nrf2), a master regulator of antioxidant gene expression (23, 164). The expression and nuclear translocation of Nrf2 are decreased in diabetic dermal fibroblasts, which leads to decreased expression of CAT, NADPH dehydrogenase quinone 1 (NOQ1), glutathione reductase, and glutathione s-transferase in response to oxidative stress (23). In fibroblasts cultured in high glucose, Nrf2 is retained in the cytoplasm by its regulator Keap1, and transcription of MnSOD and NOQ1 is reduced (164). The activity of other transcription factors is similarly altered in hyperglycemia, including AP-1 and NF-κB, which also regulate transcription of antioxidant enzymes (118, 145, 196). The role of these transcription factors in the diabetic environment should be further explored.

Redox Modulation of ECM

Oxidative stress in diabetic wounds has major implications for the ECM, and thus the progression of wound healing. Excessive ROS can alter ECM structure and composition through modulation of wound fibroblast function, direct oxidative damage, and changes in gene expression and matrix remodeling (Fig. 7).

Fibroblast function

Redox signaling is an essential mediator of fibroblast functions critical to wound healing, including proliferation, migration, ECM production, and contraction. Treatment with low levels of ROS (*i.e.*, 100 μM H_2O_2) stimulates fibroblast proliferation through activation of JNK and p38 MAPK; a similar effect is observed with partial inhibition of Cu/ZnSOD, indicating that intracellular ROS also influence cell proliferation (89). ROS production is also required for TGF-β1-induced ECM expression and fibroblast migration in response to bFGF stimulation (36, 158, 193). In fact, siRNA knockdown of the Nox2 subunit of NADPH inhibits proliferation, migration, and expression of collagen I, FN, bFGF, and PAI-1 in human dermal fibroblasts (193). In addition, Nox4 expression is required for the differentiation of fibroblasts to myofibroblasts, a transition that facilitates ECM expression and contraction (36, 83, 193).

Conversely, high levels of ROS have negative effects on fibroblast function. Fibroblasts treated with 500 μM H_2O_2 proliferate at a lower rate than untreated fibroblasts, as do fibroblasts lacking SOD3, which exhibit higher levels of intracellular ROS than control cells (63, 89). Oxidative stress can induce apoptosis in fibroblasts and particularly affects those stimulated to proliferate in wound healing assays (41, 152, 168). Increases in intracellular ROS can also lead to cellular senescence in fibroblasts, which prevents fibrosis in normal healing but may be detrimental in diabetes (85, 90). Furthermore, fibroblast migration in wound healing assays is inhibited by increased mitochondrial production of O_2^-, and is correspondingly increased with antioxidant treatment (84, 111). High ROS can also interfere with fibroblast contractile function, evidenced by reduced collagen gel contraction in cells treated with curcumin (151). Although these ROS-mediated changes in fibroblast function have been identified, the underlying mechanisms remain poorly understood. Further research could inform the development of cell-based therapeutics to improve wound healing in high-ROS environments.

ECM production

Although low levels of ROS facilitate ECM synthesis, a redox imbalance as is observed in diabetes can interfere with normal ECM production. Treatment with high concentrations of H_2O_2 (>150 mM) reduces the amount of connective tissue, and particularly collagen levels, in mouse wounds and retards wound closure (105). Similarly, treatment of fibroblasts with H_2O_2 or the SOD inhibitor diethyldithiocarbamic acid decreases both fibrillar and nonfibrillar collagen synthesis *in vitro* (162). Moreover, high levels of ROS can interfere with rather than support TGF-β1 signaling by reducing expression of the type II TGF-β receptor and Smad3 transcription factor in dermal fibroblasts (77). ROS exposure also increases

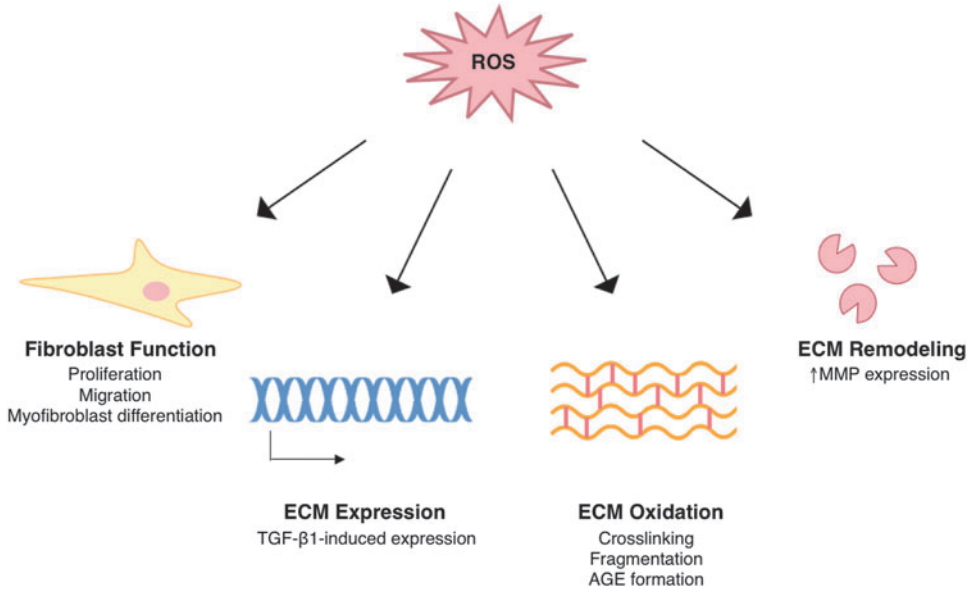

FIG. 7. Redox modulation of ECM. Redox signaling regulates ECM structure during normal wound healing, and excess ROS can cause pathological changes in ECM structure and function. TGF-β1, transforming growth factor-β1. To see this illustration in color, the reader is referred to the web version of this article at www.liebertpub.com/ars

expression of cysteine-rich protein 61 (CCN1), a negative regulator of collagen I production, in dermal fibroblasts and human skin (137).

Direct oxidative damage

The ECM is particularly susceptible to oxidative damage, even in normal conditions, because of low levels of antioxidant enzymes in the intercellular space (142). ROS-mediated protein damage is a result of oxidation of amino acid side chains, and most amino acids are easily modified by radical ROS, particularly cysteine and methionine (142). Oxidation of these amino acids can result in the formation of disulfide bridges and protein adducts, which interfere with protein structure and function (142). ROS can also significantly damage proteoglycans present in the ECM (86).

Oxidation of collagen disrupts its triple helical structure and induces inappropriate inter- and intramolecular crosslinking (54, 142). Such crosslinking can cause the formation of collagen aggregates and increase resistance to degradation by MMPs (69, 142). Oxidation can also cause protein cleavage at proline residues, leading to fragmentation of collagen, FN, and glycosaminoglycans (43, 44, 51, 138). Most studies of ECM oxidation focus solely on individual components, so the effects on overall ECM structure are not well understood.

ROS also mediate AGE accumulation in the ECM (20, 86, 127, 142). Elevated ROS in diabetes favor the formation of AGE by inhibiting GAPDH activity, and causing the accumulation of glyceradehyde-3-phosphate (G3P), a glycolysis intermediate upstream of GAPDH. G3P can be nonenzymatically converted to methylglyoxal, a highly reactive and abundant intracellular AGE precursor. ROS also contribute to the formation of AGE through both glycation and oxidation reactions; these glycoxidation products include pentosidine and carboxymethyllysine, which are among the best studied AGE. These species can further glycate collagen, elastin, and FN in the ECM (69). Like direct oxidative damage to the ECM, glycation alters mechanical proteins and cellular interactions, as already discussed (see *ECM glycation in diabetes* section).

MMP expression

Redox signaling also regulates the expression of MMPs, and thus influences ECM remodeling. ROS generation is required for the expression of MMP-1, MMP-2, and MMP-3 in human dermal fibroblasts exposed to UV light, but does not influence the expression of TIMPs (32, 76). Direct treatment with H_2O_2 also induces expression of MMP-1 in human fibroblasts *in vitro*; a similar effect is achieved by inhibition of CAT or glutathione peroxidase, enzymes that detoxify H_2O_2 (31). Notably, no change in MMP-1 was observed when Cu/ZnSOD was inhibited, indicating that H_2O_2 exerts a specific effect on the signaling pathway (31). Similarly, H_2O_2 treatment increases MMP-8 expression in granulation tissue in diabetic wounds, although the mechanism of this effect is unknown (105).

Redox-Based Wound Therapy

Treatment of diabetic wounds is largely limited to standard wound care practices, including surgical debridement, antibiotic treatment, moisture dressing, and pressure off-loading, as well as close management of blood glucose levels (171). Recent advances have focused on specific defects in the diabetic wound environment, including topical application of growth factors, introduction of bone marrow-derived endothelial and epithelial cells, and collagen-based tissue-engineered grafts (171). Notably, research has also focused on modulating the redox environment of the diabetic wound; such approaches will be reviewed hereunder.

The efficacy of altering ROS levels to improve healing in diabetes has been well established in a variety of preclinical studies. Many of these target ROS-generating mechanisms. For example, decreasing the activity of XO by topical application of an siRNA targeting its precursor, xanthine dehydrogenase (XDH), significantly improves healing in db/db diabetic mice (180). Wounds treated with siXDH exhibited a dramatic reduction in ROS levels and healed 7 days sooner than those treated with scramble siRNA control (180). Similarly, genetic deletion of the H_2O_2-generating enzyme p66Shc in diabetic mice decreased concentration of nitrotyrosine (a marker of oxidative stress) and improved healing rate, with increased granulation tissue thickness and collagen deposition as well as reduced apoptosis in the wound bed (58). Notably, topical treatment with galectin-1, which increases ROS generation through Nox, improved healing in a diabetic mouse model (101). However, diabetic wounds were not the focus of the study, and, therefore, were not extensively characterized.

Increasing antioxidant capacity has also proven to be an effective strategy; *in vivo* transfer of MnSOD improved healing rate by nearly 15% in streptozotocin (STZ)-induced diabetic mice (109). These mice exhibited increased MnSOD activity and decreased levels of O_2^- in addition to the rapid reduction of wound area (109). This study also demonstrated that increased NO availability further improved healing, but this is difficult to interpret in the context of redox signaling because NO has both oxidant and antioxidant properties [reviewed in Refs. (108, 184, 186)]. Analogously, restoration of signaling through Nrf2 accelerated healing in db/db diabetic mice (164). Nrf2 signaling was improved through topical application of siRNA for Keap1, the regulatory protein that sequesters Nrf2 in the cytoplasm. This treatment improved expression of Nrf2 target antioxidant genes, including NQO1, HO-1, glutathione reductase, and glutathione s-transferase, in the wound and improved healing time by 9 days (164). Even supplemental growth factor treatment may influence healing through ROS; topical PDGF was recently shown to increase levels of small-molecule antioxidants in the diabetic wounds, although the mechanisms must be studied more in depth (70).

Nonspecific methods of reducing ROS have also been explored recently. Topical treatment with vitamin C, a dietary antioxidant, improved wound closure at days 7 and 14 postwounding in STZ diabetic rats (96). The wounds exhibited increased collagen deposition, based on Masson's trichrome staining and hydroxyproline assays, as well as reduced apoptosis (96). Topical application of 0.3% bilirubin ointment, which scavenges ROS at low concentrations, has also been shown to improve closure rate and collagen deposition in diabetic wounds (140). Bilirubin-treated wounds also had lower MMP-9 expression and increased TGF-β1 expression relative to controls (140). Similar effects were observed with oral administration of antioxidant; 12 weeks of

treatment with the mitochondria-targeted antioxidant SkQ1 improved granulation tissue deposition—the collagen was more mature and organized and blood vessel density significantly increased (48). SkQ1-treated mice also had a greater number of α-smooth muscle actin-positive fibroblasts (48). Comparable improvements in healing were observed with SkQ1 treatment in aged mice (47). Systemic treatment with antioxidant (*N*-acetyl cysteine) also improved healing in an incisional wound model (5).

Antioxidant-based therapy is just beginning to be tested in the clinic. For example, oral administration of the polyphenol antioxidant resveratrol (RSV) was used in diabetes patients with newly diagnosed ulcers in addition to standard wound management techniques (17, 187). Patients treated with RSV showed significant improvement in ulcer size relative to the control group after 60 days, and there is evidence that RSV may decrease MMP expression and increase fibroblast proliferation *in vitro* (16, 17). However, the study was small (only 24 patients) and ECM-related parameters were not measured, so few conclusions about the efficacy of the treatment or its mechanism of action can be drawn. When combined with the successful preclinical models already described, this promising clinical data demonstrate the value of redox-based therapeutics for wound healing in diabetes. There must be further development of current antioxidant treatment strategies and evaluation of new targets to address imbalances in redox signaling in diabetes.

Conclusions

It has been recently demonstrated that ROS are critical to wound healing, and redox imbalance significantly influences ECM production and remodeling. Oxidative stress is also a critical cause of diabetic complications, including impaired wound healing. Comparison of the literature on these topics reveals several overlapping pathological mechanisms, including fibroblast dysfunction, reduced collagen deposition, oxidative damage, and dysregulated remodeling by MMPs. Given this intersection, attention must be paid to the role of ROS in diabetic wound healing. Further study of the sources and consequences of oxidative stress in the diabetic wound, with particular focus on the ECM, may allow for the development of ROS-based therapies for chronic diabetic ulcers. Based on the success of preclinical studies on antioxidant treatment, this may represent a novel and effective strategy to improve healing and prevent limb loss in diabetes patients.

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Abbreviations Used

AGE = advanced glycation end products
AP-1 = activator protein 1
bFGF = basic fibroblast growth factor
CAT = catalase
CCN1 = cysteine-rich protein 61
ECM = extracellular matrix
FN = fibronectin
G3P = glyceradehyde-3-phosphate
GAPDH = glyceraldehyde 3-phosphate dehydrogenase
GSH = glutathione
IL-1β = interleukin-1
IL-6 = interleukin-6
JNK = c-Jun n-terminal kinase
MAPK = mitogen-activated protein kinase
MMP = matrix metalloproteinase
NF-κB = nuclear factor kappa-light-chain-enhancer of activated B cells
NO = nitric oxide
NOQ1 = NADPH dehydrogenase quinone 1
Nox = NADPH oxidase
Nrf2 = nuclear factor erythroid 2-related factor 2
PDGF = platelet-derived growth factor
PKC = protein kinase C
RAGE = receptor for advanced glycation end products
ROS = reactive oxygen species
RSV = resveratrol
SOD = superoxide dismutase
STZ = streptozotocin
TGF-β1 = transforming growth factor-β1
TIMP = tissue inhibitor of metalloproteinase
TNF = tumor necrosis factor
TUNEL = terminal deoxynucleotidyl transferase dUTP nick end labeling
VEGF = vascular endothelial growth factor
XDH = xanthine dehydrogenase
XO = xanthine oxidase