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Review article

Current understanding of molecular and cellular mechanisms in fibroplasia and angiogenesis during acute wound healing

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ABSTRACT

Cutaneous wound healing ultimately functions to facilitate barrier restoration following injury-induced loss of skin integrity. It is an evolutionarily conserved, multi-cellular, multi-molecular process involving co-ordinated inter-play between complex signalling networks. Cellular proliferation is recognised as the third stage of this sequence. Within this phase, fibroplasia and angiogenesis are co-dependent processes which must be successfully completed in order to form an evolving extracellular matrix and granulation tissue. The resultant structures guide cellular infiltration, differentiation and secretory profile within the wound environment and consequently have major influence on the success or failure of wound healing. This review integrates *in vitro*, animal and human *in vivo* studies, to provide up to date descriptions of molecular and cellular interactions involved in fibroplasia and angiogenesis. Significant molecular networks include adhesion molecules, proteinases, cytokines and chemokines as well as a plethora of growth factors. These signals are produced by, and affect behaviour of, cells including fibroblasts, fibrocytes, keratinocytes, endothelial cells and inflammatory cells resulting in significant cellular phenotypic and functional plasticity, as well as controlling composition and remodelling of structural proteins including collagen and fibronectin. The interdependent relationship between angiogenesis and fibroplasia relies on dynamic reciprocity between cellular components, matrix proteins and bioactive molecules. Unbalanced regulation of any one component can have significant consequences resulting in delayed healing, chronic wounds or abnormal scar formation. Greater understanding of angiogenic and fibroplastic mechanisms underlying chronic wound pathogenesis has identified novel therapeutic targets and enabled development of improved treatment strategies including topical growth factors and skin substitutes.

Contents

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1. Introduction

A wound comprises a break in skin epithelial continuity and is characterised by disruption of structure and function of underlying tissues [1]. After injury, skin integrity must be restored promptly in order to re-establish homeostatic mechanisms, prevent infection and minimise fluid loss [2,3]. This is achieved through wound healing which describes a complex biological process where multiple parallel and interrelated pathways are activated and synchronised to induce wound repair [2,4]. Once complete, they must be shut down in a precise order to prevent exaggerated or delayed responses [5,6]. Traditionally acute wound healing is divided into 4 overlapping phases known as haemostasis, inflammation, cellular proliferation and remodelling (Fig. 1). The proliferative phase involves numerous important cellular and molecular components that contribute to extracellular matrix (ECM) and granulation tissue formation (Table 1). ECM, produced as a result of fibroplasia, provides a scaffold and signals for cellular adhesion and migration during tissue restoration and ultimately the architecture of the healed wound [7]. Angiogenesis is essential to replace damaged capillaries and restore the supply of oxygen, blood constituents and nutrients to wounded tissue, helping to return normoxia and promote fibroplasia [8]. This review provides an update on cellular and molecular mechanisms crucial to fibroplasia and angiogenesis. Furthermore, it describes mechanisms within these processes that may become deranged resulting in delayed healing or chronic wounds.

2. Overview of the proliferative phase of wound healing

Cellular proliferation represents the third phase of the 4-stage acute wound healing model [9,10]. Proliferation of cellular and structural components is triggered by factors secreted during the preceding inflammatory phase. It begins 3–4 days after injury and continues for 2–4 weeks. During this time there is fibroblast and endothelial cell proliferation, phenotypic alteration and migration as well as ECM deposition and granulation tissue formation [3,8]. Fibroblast derived ECM provides support for further cellular influx, adhesion and differentiation [4,11]. After its formation, ECM undergoes continuous synthesis and remodelling, reaching a steady state 21 days after wounding [5]. Initially disorganised fibrin is later remodelled with hyaluronan, proteoglycans and fibronectin (FN) before a predominantly collagenous final structure (mostly types I and III) is formed [11–13]. Remodelling is achieved by specific matrix metalloproteins (MMPs) influenced by transforming growth factor-β (TGF-β), platelet derived growth factor (PDGF), interleukin-1 (IL-1) and epidermal growth factor (EGF), which are tightly regulated. Blood vessel sprouts invade the wound concurrent with fibroblast in-growth. Neovascularisation occurs in response to pro-angiogenic factors including vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), angiogenin, angiotropin, and angiopoietin-1 (Ang-1) released by infiltrating macrophages and keratinocytes [14–16]. The dense population of fibroblasts, macrophages and neovasculature, embedded within a loose matrix of collagen, FN and hyaluronic

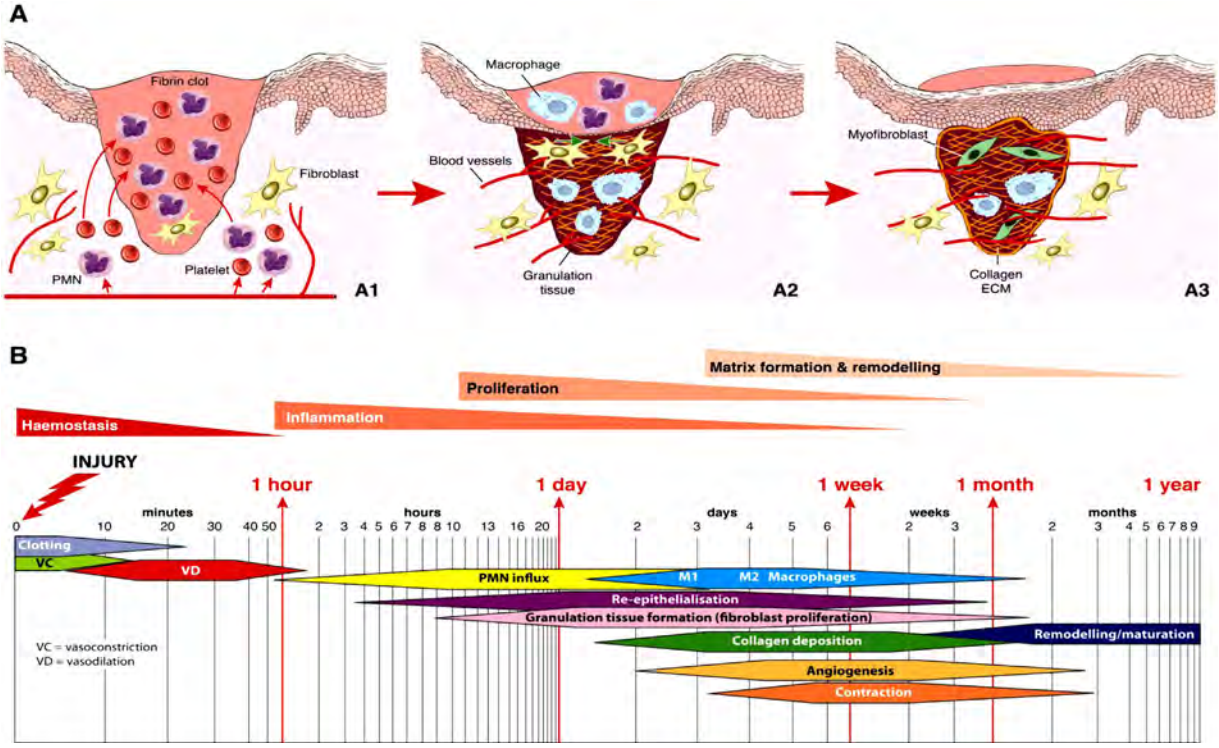

Fig. 1. Overview of acute wound healing. (A) Cellular influences in acute wound healing; (B) overlapping 4-phase model of acute wound healing and its timeframe. Phase 1 – haemostasis. After cutaneous injury vasoconstriction, clotting cascades and platelets act together to prevent prolonged haemorrhage. Once fibrin clot forms there is vasodilation enabling PMN extravasation and migration to the wound site; phase 2 – inflammation (Fig. A1). An initial influx of neutrophils is later replaced by macrophages which have an early inflammatory (M1) and later reparative (M2) phenotype. There is phagocytosis of bacteria and wound debris with concurrent secretion of multiple growth factors, chemokines and cytokines which drive fibroblast and endothelial cell recruitment to the wound bed; phase 3 – cellular proliferation (Fig. A2). Migratory endothelial cells and fibroblasts proliferate resulting in ECM deposition, angiogenesis and granulation tissue formation. ECM forms a scaffold for further cellular influx and is remodelled by a variety of enzymes so that its composition changes throughout wound healing; phase 4 – remodelling (Fig. A3). Wound maturation occurs with continued ECM remodelling into a predominantly collagenous structure, fibroblast differentiation into myofibroblasts, wound contraction and gradually reducing cellularity with eventual scar formation. Re-epithelialisation occurs concurrently beginning within hours of injury until there is restoration of epithelial continuity. PMN, polymorphonuclear cells; ECM, extracellular matrix.

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Table 1
Correlation of the main wound repair molecules involved in fibroplasia and angiogenesis, their cellular source of origin and subsequent physiological effects during wound healing.

Molecules	Cellular source	Phase of wound healing	Other physiological effects during wound healing	Reference
IL-1	Neutrophil, monocyte, macrophage, keratinocyte	Fibroplasia	Leucocyte chemotaxis, expression of growth factors, ECM degradation	[136–140]
TNF-α	Neutrophil, monocyte, macrophage, keratinocyte, mast cell	Fibroplasia	Expression of growth factors, leucocyte chemotaxis, ECM degradation, keratinocyte migration	[136,137,139–144]
EGF	Platelet, macrophage, keratinocyte	Fibroplasia	Re-epithelialisation, ECM degradation	[145–147]
TGF-β	Platelet, macrophage, fibroblast, keratinocyte, mast cell	Fibroplasia and angiogenesis	Leucocyte recruitment	[31,137,143,148–151]
HGF	Fibroblast	Angiogenesis	Re-epithelialisation, leucocyte recruitment	[152,153]
FGF-2	Platelet, keratinocyte, fibroblast, fibrocyte, macrophage, endothelial cell	Fibroplasia and angiogenesis	Endothelial cell proliferation, ECM regulation *via* MMP-1 up-regulation	[24,142,150,154–156]
PDGF	Platelet, macrophage, fibrocyte	Fibroplasia	Leucocyte recruitment	[30,137,154,156,157]
CTGF	Neutrophil, monocyte, platelet, γδ-T cell, fibroblast	Fibroplasia and angiogenesis	Scarring	[136,137,149,158–160]
IGF-1	Platelet, fibroblast, keratinocyte, DETC, BM-MSC	Fibroplasia	Keratinocyte pro-survival	[137,154,161,162]
VEGF	Macrophage, platelet, endothelial cell, keratinocyte, fibroblast, myofibroblast, fibrocyte, mast cell	Angiogenesis	Vascular permeability, endothelial cell migration and proliferation	[74–82,87,91–93]
Ang-1	Fibroblast, myofibroblast	Angiogenesis	Blood vessel stabilisation, endothelial cell proliferation	[96,97,99,163]
Ang-2	Fibroblast, myofibroblast	Angiogenesis	Antagonist to Ang-1	[96,97,99,163]
MMP-2	Dermal fibroblast	Fibroplasia and angiogenesis	ECM/collagen degradation enabling endothelial cell migration	[70,164]
uPA	Endothelial cell, keratinocyte, monocyte, macrophage, fibroblast	Fibroplasia and angiogenesis	Dissolution of fibrin, degradation of ECM, activation of growth factors and MMPs	[72,160,165–168]
tPA	Keratinocyte	Fibroplasia and angiogenesis	Dissolution of fibrin, degradation of ECM, activation of growth factors and MMPs	[165,169]
PAI-1	Migrating epidermal keratinocyte, fibroblast	Fibroplasia and angiogenesis	PA (uPA, tPA) activity regulator, keratinocyte migration	[166,170–172]

IL, interleukin; ECM, extracellular matrix; TNF-α, tumour necrosis factor-α; EGF, epidermal growth factor; TGF-β, transforming growth factor-β; HGF, hepatocyte growth factor; FGF-2, fibroblast growth factor-2; PDGF, platelet derived growth factor; CTGF, connective tissue growth factor; IGF-1, insulin-like growth factor; VEGF, vascular endothelial growth factor; Ang, angiopoietin; MMP-2, matrix metalloproteinase-2; uPA, urokinase plasminogen activator; tPA, tissue plasminogen activator; PAI-1, plasminogen activator inhibitor; PA, plasminogen activator; DETC, dendritic epidermal T cell; BM-MSC, bone marrow mesenchymal stem cell.

acid is termed granulation tissue. It is a response to abundant growth factors (GFs) and cytokines found in the wound from 4 days post-injury onwards [5,17,18]. In later stages of the proliferative phase, fibroblasts assume the myofibroblast (MF) phenotype which contains α-smooth muscle actin (αSMA) [4,19]. This enables wound contraction from day 6 post injury but is GF-dependent relying heavily on PDGF and TGF-β [7].

3. Fibroplasia

The process involving fibroblasts and the ECM they synthesise is known as fibroplasia [11]. It is influenced by numerous bioactive molecules secreted by various cell types present in the wound bed during healing (Fig. 2). GFs (especially PDGF-BB, FGF-2 and TGF-β) interacting with FN, fibrinogen chains (Aα1, Aα2 and Bβ) and thrombin in the early haemostatic clot stimulate fibroblasts to proliferate and express specific integrin receptors (Fig. 3) [20,21]. This facilitates fibroblast migration along provisional ECM fibrils into the wound space [22]. Chrissouli et al. showed that adult normal human dermal fibroblasts (NHDF) *in vitro* undergo enhanced DNA synthesis and proliferation following treatment with human amniotic fluid *via* an FGF-2 and PDGF dependent mechanism after addition of specific inhibitors significantly reduced fibroblast proliferation [23]. FGF-2 and PDGF-mediated mitogenic effects occur *via* activation of MEK/ERK and phosphatidylinositol 3-kinase (P13K)/Akt signalling pathways [23]. Furthermore, addition of exogenous TGF-β1 in cultured NHDF stimulated proliferation by activating the MEK/ERK pathway. Indeed, FGF-2 specific neutralising antibodies inhibited both ERK and TGF-β1-induced mitogenic activity [24]. Consequently, TGF-β has an autocrine mechanism of action, involving an FGF-2 loop, resulting in fibroblast proliferation [24]. Addition of recombinant FGF-2 to cutaneous wounds in animal models promoted healing with increased granulation tissue formation, containing large numbers of fibroblasts, macrophages, and capillaries in comparison to FGF-2 knock-out mice which showed delayed repair responses [25–28].

There is increasing evidence that fibrocytes contribute to new fibroblast and MF populations during normal and deranged wound healing. Prior to differentiation, immature fibrocytes secrete ECM-degrading enzymes, including MMP-2, MMP-7, MMP-8 and MMP-9 which promote fibrocyte migration into granulation tissue (MMP-8 predominant) and endothelial cell invasion (MMP-9 predominant) [29,30]. Secondary lymphoid Chemokine, a ligand for the fibrocyte chemokine receptor-7, is a potent stimulus for human fibrocyte chemotaxis *in vitro* [31]. It also promotes migration to sites of injury *in vivo* when injected into cutaneous mouse wounds [31]. Exogenous TGF-β1 stimulates *in vitro* differentiation and synthetic activity of cultured human fibrocytes into mature fibroblasts or MFs [31]. Lower concentrations of αSMA+ cells can be detected in un-stimulated fibrocyte cultures, arising secondary to low-level autocrine TGF-β1 activation [31–33].

Fibrocytes from healthy subjects and burns patients show reduced capacity *in vitro* to synthesise collagen when compared with dermal fibroblasts [34]. However, conditioned media from cultured fibrocytes of burns patients can induce dermal fibroblasts to collectively up-regulate collagen production, increase chemotactic and proliferative activity as well as differentiate into MFs [34]. The main fibrocyte secreted-cytokines responsible include TGF-β1 and connective tissue growth factor (CTGF) [34]. In addition to representing a systemic source of pre-cursor cells capable of differentiating into mature mesenchymocyte lineage,

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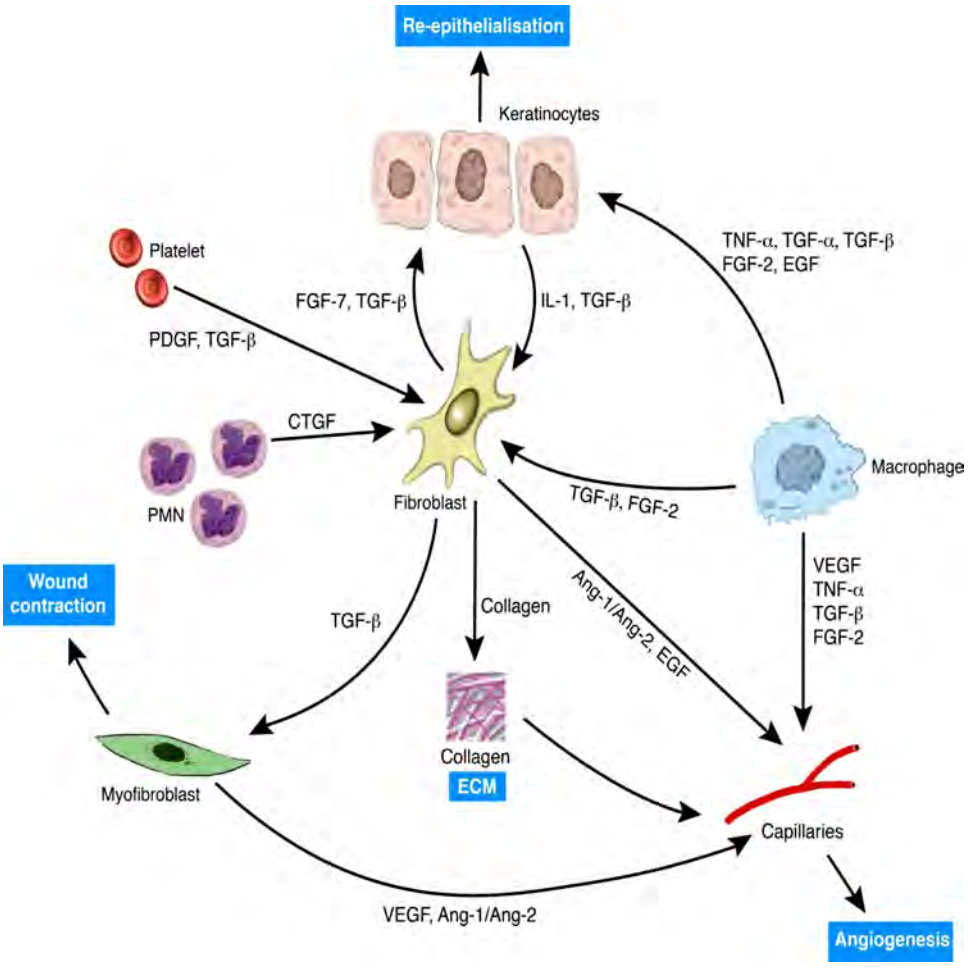

Fig. 2. Reciprocal interaction of various cell types with wound bed fibroblasts significantly influences multiple wound healing processes. Cellular influences upon wound bed fibroblasts are exerted *via* growth factors, cytokines and chemokines resulting in ECM deposition, phenotypic differentiation and establishment of regulatory feedback loops. In turn fibroblasts exert reciprocating effects upon other cell types with important roles in angiogenesis, granulation tissue formation, re-epithelialisation and wound contraction. CTGF, connective tissue growth factor; TNF-α, tumour necrosis factor; IL, interleukin; FGF, fibroblast growth factor; TGF-α/β, transforming growth factor-α/β; PDGF, platelet derived growth factor; Ang, angiopoietin; EGF, epidermal growth factor; ECM, extracellular matrix; VEGF, vascular endothelial growth factor.

fibrocytes also indirectly regulate resident fibroblast activity during wound repair [32].

Once in the wound space, fibroblasts commence synthesis of collagen-rich ECM gradually replacing provisional fibrin matrix. Collagens and FN are necessary to fill tissue defects, increase tissue tensile strength and prepare the matrix framework for new vessel formation [35,36]. Integrin expression in fibroblasts migrating into ECM changes over time, with increased α3β1 and α5β1 subunit expression initially, correlating with FN-rich provisional ECM. In contrast, later collagen deposition results in α2β1 subunits being preferentially expressed [21]. This change facilitates efficient fibroblast migration through ECM and demonstrates that ECM proteins induce expression of integrins to which they can bind. Furthermore, re-epithelialising keratinocytes induce a paracrine-mediated anti-fibrotic effect in fibroblasts [37]. Paracrine-induced fibroblasts catabolically modulate ECM *via* up-regulated urokinase-type plasminogen activator (uPA), MMP-1, and MMP-3, while down-regulating pro-fibrotic CTGF, collagen I, collagen III, FN, plasminogen activator inhibitor-1, tissue inhibitor of metalloproteinases (TIMP)-2&3 and α-SMA [37].

In vitro, co-culture of NHDF and human dermal microvascular endothelial cells (HDMEC), suggested high TGF-β1 also enhances NHDF differentiation into myofibroblasts (MFs), where levels of endothelin-1 (ET-1) and wound tissue oxygen are low [36]. MFs also arise from extra-domain-A fibronectin (EDA-FN) splice-variants expressing proto-MFs and stellate cells, in response to elevated TGF-β1 and increased wound tension [38,39]. Fibroblasts themselves generate tractional forces *in vitro*, possibly sufficient to initiate wound closure [40]. However, contraction is considered to formally commence once MF differentiation has occurred, 10–14 days after initial injury (Fig. 4) [39,41,42].

Differentiated MFs are distinguished morphologically from proto-MFs by raised EDA-FN levels, *de novo* α-SMA expression, increased stress fibre assembly and focal adhesions [39]. Indeed, elevated MF derived α-SMA has previously been correlated with acquisition of contractile phenotype and force generation [43]. However, MFs express other smooth muscle contractile proteins including h1-calponin and γ-SMA [44,45]. Tomasek et al. demonstrated that full thickness excisional wounds in α-SMA-null mice healed at similar rates to wild-type mice generating similar amounts of focal adhesions, stress fibres and contractile force [46]. This suggests that proteins including γ-SMA can functionally compensate in the absence of α-SMA in order to facilitate MF differentiation and function in knock-out models whilst the mechanical protein mediating the contractile phenotype of wound MFs remains elusive [46].

In vitro stress-relaxed collagen lattice contraction assays demonstrated TGF-β1 promoted dose-dependent increases in MF contractile force with concomitant increases in α-SMA-containing stress fibres [47]. Therefore MFs populate the wound

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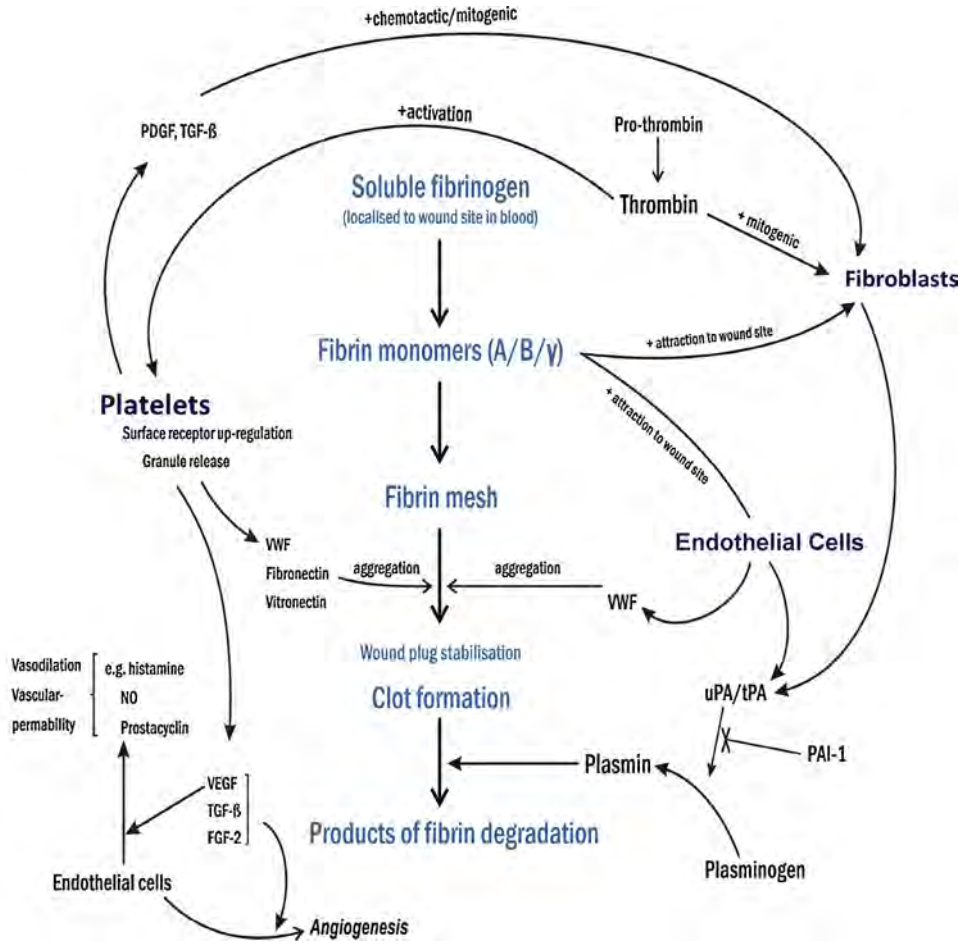

Fig. 3. The role of clot formation after cutaneous injury in the activation of fibroblasts and endothelial cells resulting in subsequent fibroplasia and angiogenesis. Cutaneous injury results in activation of haemostatic mechanisms to limit blood loss and provide a framework for later stages of wound healing. Formation of the fibrin clot results in activation and migration of surrounding fibroblasts and endothelial cells through interaction with Aα and Bβ chains of fibrin monomers [21]. Fibroblast chemotaxis and proliferation is also stimulated by platelet derived factors including PDGF, TGF-β as well as proteins such as thrombin [11]. Endothelial cells are encouraged to migrate and commence angiogenesis by platelet derived VEGF, TGF-β, and FGF-2 [154]. Once established in the wound space, fibroblasts and endothelial cells are involved in fibrinolysis through secretion of uPA and tPA to enable later tissue remodelling and migration across the extracellular matrix [165]. Endothelial cells also secrete histamine, NO and prostacyclin resulting in increased vascular permeability and vasodilation which promotes further endothelial cell migration and inflammatory cell influx [87]. VEGF, vascular endothelial growth factor; TGF-β, transforming growth factor-β; FGF-2, fibroblast growth factor-2; NO, nitric oxide; PDGF, platelet derived growth factor; TGF-β, transforming growth factor-β; vWF, Von Willebrands factor; tPA, tissue plasminogen activator; uPA, urokinase plasminogen activator.

secondary to a positive feedback mechanism, where tension facilitates TGF-β1 production, resulting in development of increased force and sustained MF function (Fig. 4) [39]. Tumour necrosis factor-α (TNF-α) suppresses TGF-β-induced expression of MF phenotypic genes by reducing TGF-β mRNA stability, with resultant reduced collagen lattice contraction *in vitro* [48]. Thus, wound tension combined with balanced cytokine levels is vital to determine ECM mechanical properties during wound repair.

MF contractile axial actin microfilaments, terminate at the fibronexus, forming an adhesion complex linking intracellular actin filaments to extracellular FN fibrils *via* transmembrane integrins [49]. This mechano-transduction system allows force generated within MFs to be transmitted to surrounding ECM [50]. Tomasek previously outlined the "slip and ratchet" hypothesis of how MF contracture translates into functional shortening of collagen-based ECM [39]. Intracellular stress-fibres of MFs bind collagen *via* linkage to fibronexus adhesion complexes. Stress-fibre contraction secondary to intracellular signalling leads to corresponding localised contraction of the collagen matrix. New matrix components are then added to stabilise newly condensed tissue. These effects are localised, dynamic and incremental. The original MF and surrounding neighbours repeat the process so that remodelling of small sections produces an overall tissue contraction response [39]. *In vitro* models by Oberringer additionally found that MFs may support HDMEC to induce angiogenesis [36]. As healing progresses into late proliferation, increased blood supply combined with increased ET-1 produced from MF cells and a constantly high TGF-β1, may induce re-activation of fibroblasts to proliferate and migrate, promoting increased cell mass and hence ECM production [36].

Fibroblasts suspended in collagen matrices *in vitro* produce reduced levels of collagen in the presence of TGF-β compared with fibrin-gels indicating collagen attenuates fibroblastic synthetic responses to TGF-β [13]. Dermal fibroblasts in detached or stress-relaxed matrices have enhanced TGF-β expression, present a spherical morphology lacking stress fibres and show elevated levels of apoptosis [51]. Thus, after abundant collagen has filled the wound site, fibroblasts stop producing collagen and fibroblast-rich granulation tissue is replaced by relatively acellular scar tissue. Akasaka et al. suggest FGF-2 is causal in reducing granulation tissue volume late in wound healing with increased apoptosis in rat FGF-2-treated wounds compared to controls in the first 14 days of healing [26]. Furthermore, apoptosis in MFs was significantly increased following FGF-2 treatment, with subsequent decreases in mature collagen bundle formation and reduced wound contracture [52,53].

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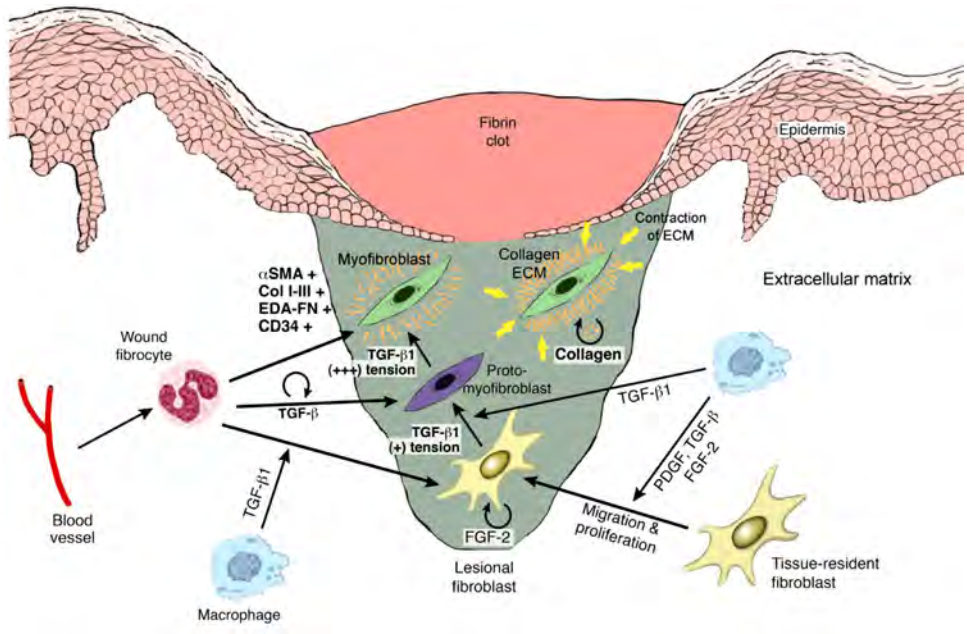

Fig. 4. Influences promoting phenotypic differentiation of fibroblasts into myofibroblasts resulting in wound contraction. Macrophage-derived PDGF, TGF-β and FGF-2 result in migration of fibroblasts from surrounding healthy tissue to the wound site where they begin the process of fibroplasia [11]. TGF-β1 can also induce bone-marrow derived fibrocyte differentiation into fibroblasts, proto-myofibroblasts and myofibroblasts [31]. Later in the proliferative phase fibroblasts convert to proto-myofibroblasts in response to increased tissue tension and TGF-β [36]. A positive feedback loop is created where tension facilitates further TGF-β release and final maturation into myofibroblasts which generate the majority of contractile forces in the wound [39]. Myofibroblasts are recognised by increased expression of αSMA, collagen I & III, EDA-FN and CD34 positive cells [39]. TGF-β, transforming growth factor-β; αSMA, α-smooth muscle actin; Col I–III, collagen types I and III; EDA-FN, extra domain A fibronectin; PDGF, platelet derived growth factor; FGF-2, fibroblast growth factor-2.

4. The influence of fibroplasia on angiogenesis

The structure and composition of ECM alters significantly during wound healing. Consequently its effect on angiogenesis is variable secondary to the protein constituents at any one time, the actions of proteases upon ECM and ECMs ability to sequester growth factors and bioactive molecular fragments [54]. ECM proteins including collagen, laminin, vitronectin and particularly FN promote endothelial cell proliferation, survival and migration [54]. FN additionally binds to VEGF and enhances VEGF-induced endothelial cell migration *via* increased mitogen activated protein kinase activity [55]. MMP and plasmin degradation of ECM can promote angiogenesis by stimulating endothelial cell migration *via* decreasing the density of ECM proteins and through exposure of pro-migratory matrix molecule binding sites [56–58]. Furthermore, protease action may release matrix-bound angiogenic factors including FGF-2 [59]. However, their action can also liberate anti-angiogenic ECM fragments including endostatin, and non-collagenous domains of various collagens [57,60]. Other intact ECM proteins are anti-angiogenic including thrombospondin-1, thrombospondin-2 and decorin [61,62]. Thrombospondin-1 inhibits angiogenesis stimulated by FGF and promotes endothelial cell apoptosis [63,64]. In conclusion, the precise composition of ECM as well as angiogenic and anti-angiogenic factors is spatially and temporally controlled during healing. The balance between them determines specific endothelial cell behaviour and vessel organisation.

5. Angiogenesis

During the proliferative phase, wound microvasculature is reconstructed in order to re-establish the nutrient supply to regenerating tissue, promote fibroplasia and prevent sustained tissue hypoxia (Fig. 5). Failure to do so will result in delayed wound healing [11,36].

Similar to the migrating epithelial tongue seen in re-epithelialisation, non-proliferating endothelial cells migrate into the wound at the leading tip of capillaries [65]. Platelet-derived factors, and ECM components including FN and heparin, promote phenotypic alterations of endothelial cells 2 days post-wounding. These include cytoplasmic pseudopodia extension, secretion of collagenases and subsequent migration into the peri-vascular space [66].

Fibrin and fibrinogen bound FGF-2 is capable of enhancing endothelial cell growth *in vitro*, with fibrin(ogen) potentiating FGF-2's proliferative activity through co-localisation of integrin αvβ3 and FGF-R1 [67,68]. This suggests that post-injury, fibrin localises FGF-2 to regulate endothelial cell responses. Indeed, αvβ3 is a defining angiogenic marker having been identified on blood vessels in human granulation tissue but absent from vessels in normal skin [69]. FGF-2 induced αvβ3 may also facilitate localisation of MMP-2 to endothelial cells, which promotes collagen degradation and subsequent endothelial migration [70]. Heparin and heparin sulphate (HepS), found in ECM, also directly enhance FGF function by enhancing association of FGF with FGFR-R tyrosine kinase in ternary complexes on endothelial cell surfaces [71].

Koolwijk and Kroon utilised 3D fibrin matrices to investigate the ability of human microvascular endothelial cells (hMVECs) to form capillary-like structures *in vitro*. Addition of FGF-2 and/or VEGF with TNF-α induced tubule outgrowth, with strongest effects seen after simultaneous addition of all three factors [72]. Furthermore, hypoxia induced a 2–3 fold increase in tube formation compared to normoxic conditions through up-regulation of uPA-R on hMVECs [73]. Thus, FGF-2 in combination with VEGF, TNF-α and hypoxia, promote increased plasmin levels, *via* uPA up-regulation which facilitates endothelial cell invasion into

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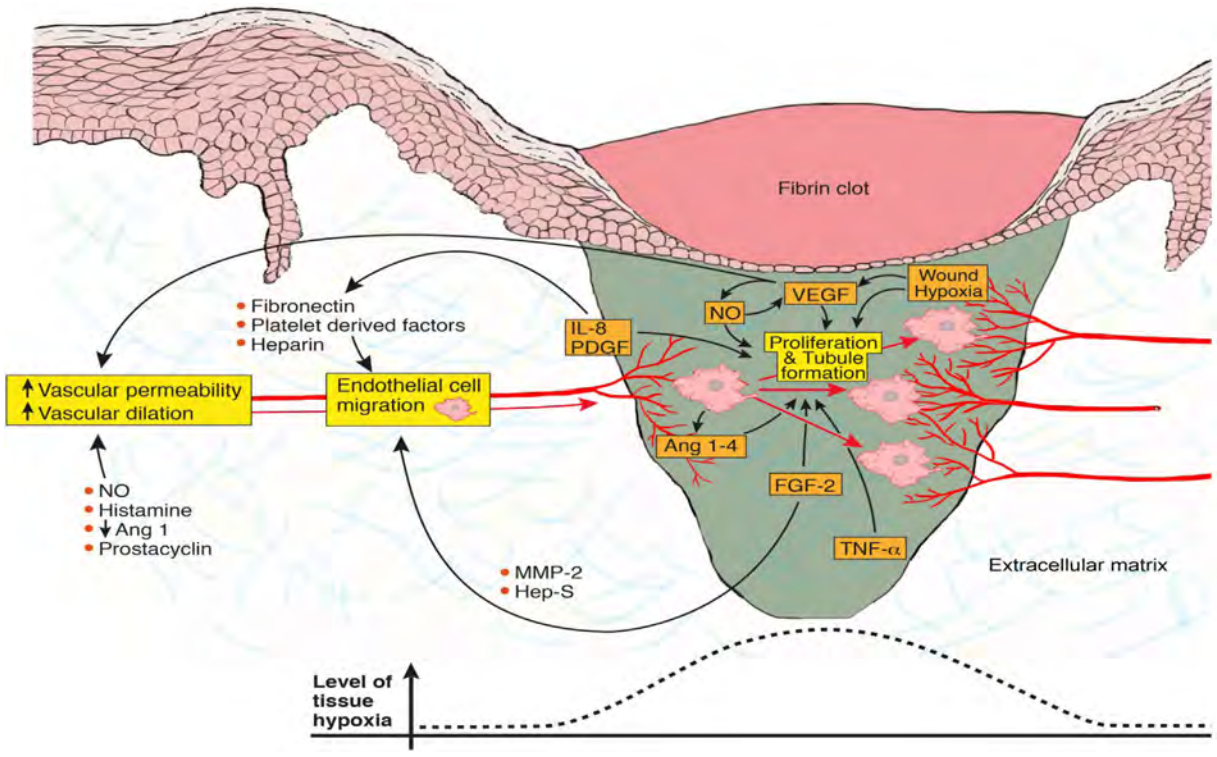

Fig. 5. Molecular and cellular factors required for angiogenesis in order to promote endothelial cell migration, proliferation and tubule formation. After wounding, angiogenesis functions to re-establish normoxia and nutrient delivery to damaged and regenerating tissue. Existing capillaries in the wound margin become dilated with increased permeability secondary to the influence of inflammatory mediators including NO, histamin, Ang-1, prostacyclin and VEGF [87,97]. This facilitates endothelial cell extravasation and migration into the perivascular space. The process is further encouraged by platelet derived factors, ECM components (heparin and fibronectin), PDGF, IL-8 and FGF-2 which may be fibrin or fibrinogen bound [66,67]. FGF-2 induced integrin expression facilitates MMP-2 localisation resulting in collagen degradation thereby facilitating endothelial cell migration [70]. Once present in the wound bed, endothelial cells proliferate and form new capillary tubules contributing to granulation tissue formation and restoration of circulatory integrity. VEGF replaces FGF-2 as the predominant stimulant of this process after 7 days and is secreted by a range of cellular mediators including macrophages, neutrophils, endothelial cells, keratinocytes and fibroblasts [80]. Other important proliferative factors include tissue hypoxia, NO, TNF-α and Ang1-4 [87,94]. NO, nitric oxide; Ang-1-4, angiopoietin-1-4; VEGF, vascular endothelial growth factor; ECM, extracellular matrix; PDGF, platelet derived growth factor; IL-8, interleukin-8; FGF-2, fibroblast growth factor-2; Hep, heparin; Hep-S, heparin sulphate; MMP-2, matrix metalloproteinase-2; TNFα, tumour necrosis factor-α.

the fibrin matrix promoting endothelial cell migration and angiogenesis [69,72,73].

VEGF is vital in angiogenesis. It is predominantly released by keratinocytes during wound healing but also secreted from intracellular pools within platelets and neutrophils as well as by macrophages, endothelial cells, vascular smooth muscle cells and fibroblasts [74–80]. *In vitro* studies show VEGF binds specifically and saturably to fibrin(ogen) with high affinity, retaining its capacity to support endothelial cell proliferation when bound to both soluble or surface immobilised fibrin(ogen). This interaction facilitates localisation and mitogenic activity of VEGF at sites of tissue injury [81]. Data from 3D angiogenesis models indicates VEGF-A or VEGF-C activation of VEGF-R2 receptors, and not VEGF-R1, is involved in formation of capillary-like tubular structures within a fibrin mesh [82]. Indeed, VEGF-R2 responses to VEGF induce actin re-organisation, membrane ruffling, chemotaxis, and mitogenicity in endothelial cells. These morphological changes are lacking in cells exclusively expressing VEGF-R1 [83]. VEGF-induced effects are mediated, by up-regulation of several integrin receptors ($\alpha V\beta 3$, $\alpha 1\beta 1$, and $\alpha 2\beta 1$) and increased expression of the anti-apoptotic protein Bcl-2 [84,85].

VEGF expression is also enhanced by hypoxia in cultured human keratinocytes, HDMECs and dermal fibroblasts *in vitro* [79]. Pro-angiogenic hypoxic effects are mediated through hypoxia-inducible factor-1α (Hif), which further up-regulates VEGF, endothelial nitric oxide synthase (eNOS) and heme oxygenase-1 (HO-1) expression [86]. VEGF stimulates eNOS *via* several pathways (Akt/PKB, Ca^{2+}/calmodulin and protein kinase C) and in turn eNOS derived nitric oxide (NO) is a positive modulator of VEGF expression. Both NO-mediated VEGF expression and VEGF-mediated NO production are regulated by Hif-1 and HO-1 [87]. This establishes a VEGF gradient within the wound, correlating with a parallel hypoxic gradient, stimulating endothelial cells to migrate towards central hypoxic regions. If this gradient is removed, new vessel formation is inhibited [88]. VEGF is highly up-regulated 3–7 days post-wounding, promoting endothelial cell migration, proliferation, and enhanced vascular permeability [80]. In contrast, FGF-2 is highest after initial injury in human wound fluid and declines towards day 7, suggesting FGF-2 provides early angiogenic signals, followed by later VEGF-mediated responses [80]. Wound fibrocytes express FGF-2, VEGF, IL-8, PDGF and hematopoietic GFs, promoting *in vitro* endothelial cell migration, proliferation, and tubule formation [30].

Myeloid cell derived VEGF-A plays a critical role in angiogenesis after wounding [89]. Effective vascularisation has classically been attributed to the M2 "repair macrophage" phenotype [90]. However, conclusive evidence of macrophage polarisation and/or specific sequential macrophage phenotype requirements during acute wound healing is lacking. Willenborg et al. showed that chemokine-receptor 2 (CCR2) signalling is critical for recruitment of $Ly6C^+$ inflammatory circulating blood monocytes into cutaneous wounds that give rise to a highly proangiogenic VEGF-expressing macrophage subset resulting in induction of vascular sprouts during early phases of healing [91]. This macrophage subset is also involved in induction of MF differentiation, but has no effect on subsequent scar formation or wound closure [91,92]. Thus it is

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suspected that monocytes/macrophages initiate vasculogenesis in wounded tissue by a process requiring specific monocyte/macrophage derived VEGF. Later vascular development then requires a dynamic interplay of VEGF from other sources including epidermal cells [91]. This theory is supported by murine studies using keratinocyte restricted VEGF deficiency mice which showed disturbances in late phase vascular growth whilst early angiogenesis was unaffected [93].

Angiopoietins are endothelial cell-specific GFs, having separate but synergistic effects with VEGF on angiogenesis. Ang-1/2/3/4 bind Tie-2 receptors expressed on endothelial cells [94]. Ang-1, Ang-2 and Tie-2 are also expressed by fibroblasts and myofibroblasts in healthy human wounds [95]. Ang-1 can phosphorylate Tie-2 and has anti-apoptotic effects on endothelial cells, mediated *via* PI3K/Akt-dependent pathways [96]. *In vivo* Ang-1 overexpressed in mice leads to formation of mature vessels [97]. Indeed, when Ang-1/VEGF are co-expressed in transgenic mice, Ang-1 is capable of overcoming the leaky immature vessel effect otherwise induced by VEGF [98]. In humans, constitutive levels of Ang-1 are known to decrease soon after wounding, promoting vessel de-stabilisation and allowing enhanced vessel response to pro-angiogenic factors [99]. A subsequent Tie-2/VEGF rise produces increased micro-vessel density whilst elevated fibroblast/MF derived Ang-1/Ang-2/VEGF bind Tie-2 on vessels to augment angiogenesis and promote fibroblast differentiation to MFs [95]. Endothelial Ang-2 expression remains elevated in scar tissue promoting vascular regression supported by decreased VEGF levels [99]. Ang-1 also slowly returns to normal levels, aiding stabilisation of remaining vasculature [95].

6. The influence of angiogenesis on fibroplasia

The effects of angiogenesis and proangiogenic factors including VEGF on fibroplasia, ECM remodelling and scarring are not clear. Stimulation of angiogenesis has been shown to enhance healing rates particularly in impaired healing models such as diabetic subjects [100,101]. Other studies demonstrated that modulation of angiogenesis did not significantly affect epidermal healing or overall wound closure rates [102,103]. In contrast, Wilgus et al. showed that VEGF levels influence scar tissue formation during wound healing using models of scarless and fibrotic repair in mice [104]. Scarless foetal wounds had lower VEGF levels and were less vascular than fibrotic foetal wounds. Furthermore the scarless phenotype could be converted to a scar-forming phenotype by addition of exogenous VEGF. Reduction of VEGF produced less vascularity and decreased scar formation in adult wounds [104]. The underlying mechanism is unknown but VEGF may directly stimulate both endothelial cells and fibroblasts. Indeed Wu et al. demonstrated functional VEGF receptors on fibroblasts, after VEGF administration induced proliferation of keloid fibroblasts whilst Zhang et al. found VEGF-R2 in stromal cells during wound repair *in vivo* [105,106]. Other studies have demonstrated that angiogenic inhibitors including endostatin, CM101 and the VEGF-R2 blocker ZD6474 positively influence ECM deposition and reduce scar formation [107–109]. In contrast proangiogenic factors including PDGF, TGF-β and interleukin-8 were either present at lower concentrations during scarless foetal healing or induced scarring when added to this environment [110–112]. In conclusion, angiogenesis must be tightly regulated in order to ensure healing whilst limiting its pro-fibrotic effects.

7. What happens when these processes become deranged?

In some cases, the combination of patient specific systemic (*e.g.* diabetes, vascular insufficiency, nutritional deficiencies and increasing age) and localised tissue factors (*e.g.* excess protease activity and bacterial load) produces persistent pathological inflammation resulting in chronic wound formation [2,5,113]. A chronic wound is defined as a break in epithelial continuity of greater than 42 days duration or of frequent recurrence [114,115]. Chronic wound prevalence varies with age, being approximately 1% in the adult population but increasing to 3–5% in those over 65 years [116,117]. Actual numbers are increasing secondary to an ageing population and rising incidence of risk factors including smoking, diabetes and obesity. Management of chronic wounds including venous and diabetic ulcers is labour intensive and costly for healthcare providers. Current treatment modalities such as compression bandaging are slow to work with significant failure rates [118]. Greater understanding of mechanisms underlying chronic wound pathogenesis has enabled new effective management strategies and treatment delivery methods to be developed. These include the use of skin substitutes and topical growth factors as well as gene and stem cell therapies.

8. Mechanisms specific to the cellular proliferative phase contributing to chronic wound formation

A defining feature of chronic wounds is unbalanced proteolytic activity, which overwhelms tissue protective mechanisms [2,119–122]. Within chronic wounds, activated keratinocytes, fibroblasts, endothelial cells, neutrophils and macrophages show increased protease expression (predominantly cathepsin G, uPA-R and neutrophil elastase) [122–124]. In combination with high levels of reactive oxygen species (ROS) (produced secondary to wound bacterial bioburden), there is damage to ECM proteins and direct cell membrane injury. Furthermore ROS can selectively affect signalling pathways leading to activation of transcription factors that control expression of pro-inflammatory chemokines, cytokines (IL-1, IL-6, TNF-α) and proteolytic enzymes (MMPs and serine proteases) [2,125]. Expression and activity of various MMP classes, including collagenases, gelatinases, stromelysins and membrane type MMP are up-regulated in chronic venous ulcers [2,121,126]. Furthermore, pro-inflammatory cytokines are potent inducers of MMP expression in chronic wounds, and down-regulate TIMP expression, thereby creating an environment with excessive MMP activity. Consequently, wound repair mediators become targets of proteases. Indeed α1-proteinase inhibitor, α2-macroglobulin and ECM components (FN and vibronectin) are downgraded or inactivated within chronic wounds [123,124]. The resultant matrix degradation contributes to delays in re-epithelialisation, fibroplasia and angiogenesis [127]. Fibroblasts within chronic wounds show senescent behaviour with altered morphology, diminished migratory capacity, slower rates of proliferation and reduced responsiveness to applied GFs [128–130]. Furthermore, chronic wounds demonstrate reduced levels of GFs including FGF, epidermal growth factor (EGF), PDGF, VEGF and TGF-β compared to acute wounds [131–134]. This may be secondary to degradation by excessive proteases or trapping by ECM molecules [135].

9. Conclusion

Increasing scientific knowledge has helped define highly co-ordinated molecular and cellular events involved in fibroplasia and angiogenesis. These processes are intimately linked and governed by dynamic reciprocity involving the interaction of appropriate cell types, cell surface receptors, extracellular matrix proteins and bioactive molecules. They must be tightly regulated in order to facilitate successful healing whilst derangement can result in chronic wound formation and excessive scarring. Further understanding of mechanisms involved in fibroplasia and angiogenesis

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will continue to provide new therapeutic targets and strategies for their treatment.

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