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Chronic Wound Healing: Molecular and Biochemical Basis

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1.1 Introduction

A wound can be defined as a break in the epithelial integrity of the tissue, or a disruption of normal anatomical structure and function [1]. Usually a wound progresses through several sequential, though overlapping, stages of cellular and biochemical activity to achieve healing. A chronic wound may be defined as one that is failing to progress through the wound healing process in an anticipated time frame [2]. A wound that does not show significant improvement within 4 weeks, or heal completely in 8 weeks, may be considered a chronic wound [3]. There are four stages described in normal wound healing: haemostasis, inflammation, proliferation, and remodelling. The healing of a chronic wound may be arrested in any of these stages, but most commonly during inflammation or proliferation [4]. This chapter will briefly describe normal wound healing, consider some subtypes of chronic wound, and then examine the different molecular and biochemical processes that occur.

1.2 Acute Wound Healing

The process of acute wound healing is well described and widely reported in the literature, and is summarised in Figure 1.1.

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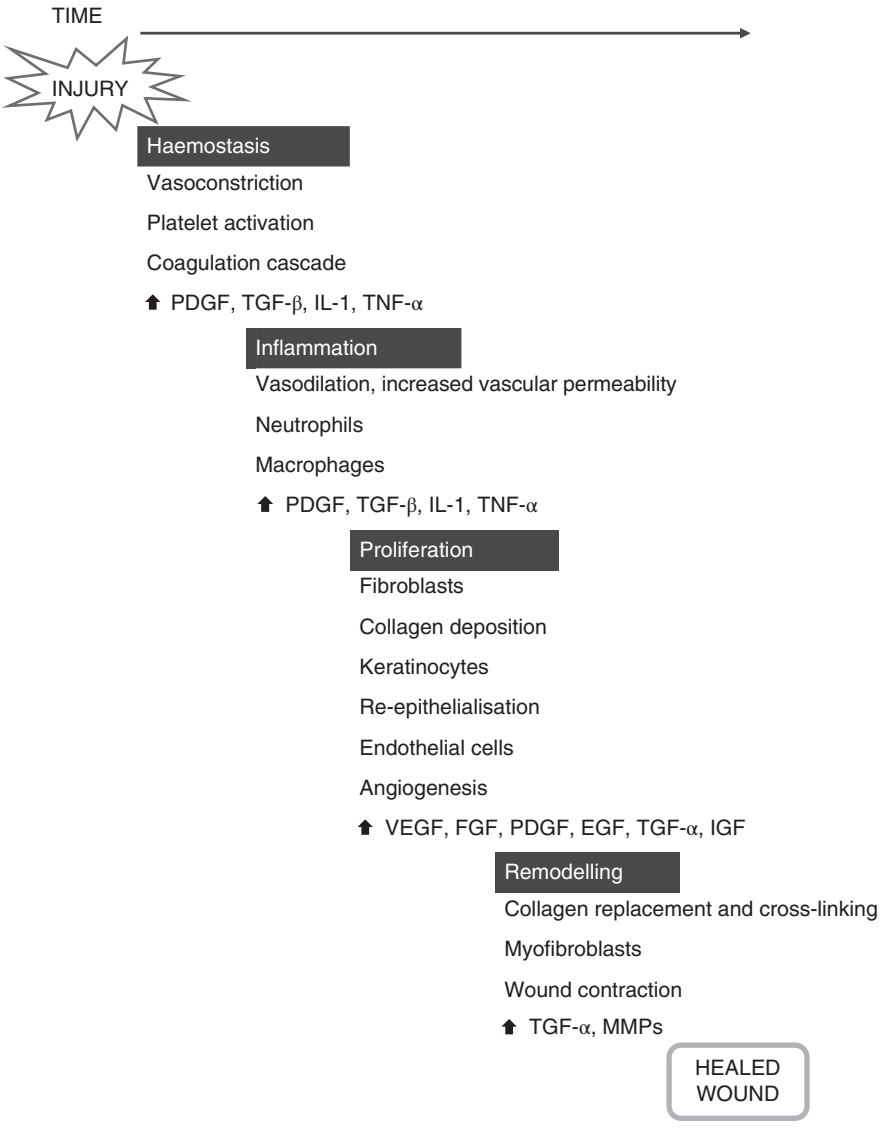

Figure 1.1 *A summary of acute wound healing. EGF, epidermal growth factor; FGF, fibroblast growth factor; IGF, insulin-like growth factor; IL-1, interleukin 1; MMP, matrix metalloproteinase; PDGF, platelet-derived growth factor; TGF, transforming growth factor; TNF-α, tumour necrosis factor α; VEGF, vascular endothelial growth factor.*

The first step, haemostasis, is characterised by vasoconstriction and coagulation; it takes place soon after injury and is complete within hours. The tissue in the wound is exposed to blood because of disruption of the blood vessels and lymphatics during injury. Platelets are activated when they come into contact with collagen and initiate the coagulation cascade, resulting in the deposition of a haemostatic 'plug' [5]. A number of cytokines are released

by the degranulation of activated platelets. Of particular importance are platelet-derived growth factor (PDGF) and transforming growth factor-beta (TGF-β). PDGF is a chemoattractant of neutrophils, macrophages, smooth muscle cells, and fibroblasts [1]. TGF-β is also involved in the chemotaxis of macrophages, fibroblasts, and smooth muscle cells, and has a role in activating these cells to express other cytokines and enzymes which are crucial to enable the wound healing to progress [1].

After the initial vasoconstriction during haemostasis, there is vasodilation and increased vascular permeability as the stage of inflammation begins. This is regulated by mast cell degranulation, which releases histamine and other vasoactive mediators [1]. Debris, dead cells, and bacteria are cleared from the tissue by neutrophils, and later by macrophages. Inflammation is usually complete after 48–72 h, but may last as long as 5–7 days [6].

The next stage is proliferation, which continues for weeks. The hallmark of the proliferative phase is the migration of fibroblasts into the wound, where they are activated to produce collagen III, fibrin, fibronectin, and hyaluronic acid in the new extracellular matrix [7]. Granulation tissue is deposited to fill the defect. Keratinocytes, stimulated by epidermal growth factor (EGF) and transforming growth factor-alpha (TGF-α), migrate to the wound edges, and eventually close the defect [1]. Angiogenesis is important to support the increased metabolic activity in the wound. A number of growth factors stimulate the neovascularisation, including vascular endothelial growth factor (VEGF). Epidermal cells, fibroblasts, macrophages, and vascular endothelial cells produce these factors in response to conditions in the wound environment, such as low pH and reduced oxygen tension [1].

The final stage, remodelling, begins after about a week and may last for years. This phase is characterised by the removal of type III collagen from the extracellular matrix and the deposition of mature type I collagen [8]. Collagenase enzymes from fibroblasts, neutrophils, and macrophages are important in this stage [1]. Wound contraction is mediated by differentiated fibroblasts (myofibroblasts) in response to TGF-α, and the presence of matrix proteins such as extra-domain-A fibronectin and tenascin C [9]. Once remodelling has occurred, there is apoptosis of fibroblasts, leaving relatively acellular scar tissue [9].

1.3 Categories of Chronic Wound

Although chronic wounds may seem varied in their presentation and characteristics, often the underlying aetiological processes are similar. Some common chronic wound categories are considered here. Ultimately, the final common pathway is an open wound that has been colonised with bacteria, initiating a damaging inflammatory response that impedes healing [10].

1.3.1 Pressure Ulcers

Pressure ulcers are an example of chronic ischaemia–reperfusion injury. Repeated tissue trauma occurs in insensate areas when the pressure in the tissue exceeds capillary perfusion pressure [10]. This results in skin breakdown, which is followed by bacterial colonisation, often compounded by the location of such ulcers near to the perineum. There is failure of the processes of angiogenesis, extracellular matrix deposition, and wound contraction, resulting in the development and persistence of a chronic ulcer [11]. These steps in wound

healing are usually driven by growth factors, and the destruction or reduced synthesis of these proteins in pressure ulcers has been investigated. In a study using an enzyme-linked immunosorbent assay technique to quantify the levels of growth factors in wound fluid from pressure ulcers, Cooper et al. [12] found that PDGF, fibroblast growth factor (FGF), EGF, and TGF-β levels were variable, and decreased compared with the levels of growth factors in acute wounds.

1.3.2 Venous Stasis Ulcers

Venous stasis ulcers occur when damaged or defective leg vein valves result in venous hypertension and oedema. Eventually the venous pressure exceeds the capillary perfusion pressure of the skin, and the tissue becomes ischaemic. The increase in intraluminal pressure affects the permeability of the vessel walls, and the veins leak fibrin and other plasma components into the perivascular space [9]. Accumulation of fibrin impairs healing by impairing collagen synthesis, and by forming peri-capillary fibrin cuffs that impede normal vessel function [9]. Often a venous ulcer is precipitated by minor trauma, for example a scratch or insect bite. The skin breakdown is accelerated by the hypoxic conditions, and secondary bacterial colonisation. This increases the tissue injury and inflammation at the wound site, and impairs epithelialisation [11].

1.3.3 Ischaemic Ulcers

Atherosclerosis and/or embolism in leg arteries leads to narrowing of the lumens of the vessels and ischaemia of distal tissue. Minor trauma may then result in an ulcer. Healing is slow because of the low oxygen concentration in the tissue, and the resultant open wound is colonised by bacteria. This increases inflammation in the wound, and the tissue defect persists. The effects of hypoxia are described in more detail in Section 1.5.4.

1.3.4 Diabetic Foot Ulcers

Diabetic foot ulcers are another category of wounds which are commonly chronic in their course. The diabetic foot may be subject to repeated trauma as a result of sensory loss. There may also be a degree of ischaemia because of microvascular arteriopathy. Once the skin barrier is breached, low-grade bacterial colonisation is common. Tissue fragments and bacterial products perpetuate the inflammatory response. The effects of hyperglycaemia are described in more detail in Section 1.5.2.

1.4 How a Chronic Wound Develops: Intrinsic Components

There are several hallmarks of chronic wounds when compared with normal acute wounds [9]. In a normal wound bed, there will be a high concentration of growth factors, with healthy cell populations in an organised extracellular matrix. By comparison, chronic wound beds tend to have low concentrations of growth factors and a disorganised extracellular matrix. This is because of excessive proteolysis driven by a persistent inflammatory state, often a response to a bacterial biofilm or low-grade infection. Impaired angiogenesis

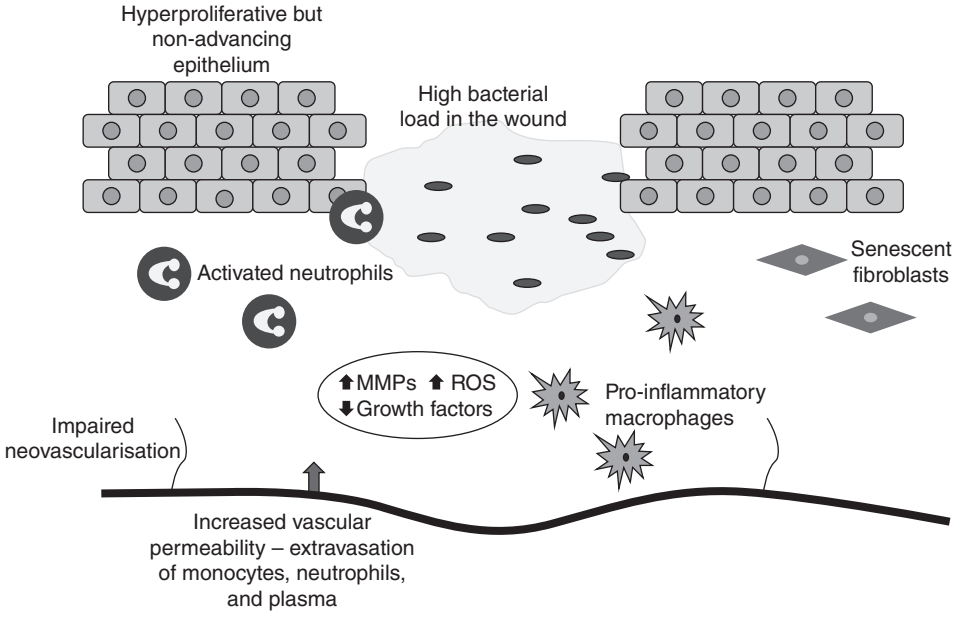

Figure 1.2 *The local environment in the chronic wound. MMP, matrix metalloproteinase; ROS, reactive oxygen species.*

and neovascularisation mean that cells in the wound environment are starved of oxygen and nutrients. The result is impaired fibroblast and epithelial cell proliferation and migration, and delayed healing. Figure 1.2 summarises these components.

1.4.1 Cell Phenotype

The cells in chronic wounds have an altered phenotype, with fewer growth factor receptors and less mitogenic potential [9]. They do not therefore respond to the wound environment in the same way as cells observed in a healthy acute wound. Some specific observations in different cell types are briefly described below.

1.4.1.1 Fibroblasts

In a healthy wound, fibroblasts respond to chemical signals in the form of growth factors, such as PDGF, insulin-like growth factor (IGF), and FGF, to migrate towards the site of injury, divide, and synthesise key extracellular matrix proteins such as collagen III, fibrin, fibronectin, and hyaluronic acid [7]. Fibroblasts from many chronic wound types have a reduced response to growth factors. Studies of fibroblasts from chronic diabetic [13], chronic non-diabetic [13], and chronic venous ulcers [14] have demonstrated lower rates of cell division in response to PDGF, IGF, and FGF, which usually promote proliferation, while cell motility is also reduced at the same time. These findings are thought to be due to reduced growth factor receptor density [9]. As well as having reduced activity, fibroblasts from chronic wounds show signs of premature senescence. In a study where fibroblast

cultures were generated from punch biopsies taken from venous leg ulcers, and compared with those from uninjured skin in the contralateral limb, the cells from the wound showed a reduction in growth potential and altered gene expression. This observation was independent of the age of the patient [15].

1.4.1.2 Keratinocytes

In healthy skin, basal keratinocytes at the dermal–epidermal junction undergo cell division periodically, and then these daughter cells differentiate to form the supra-basal epidermal layers. Keratinocytes express keratin proteins, with the pattern of keratin filament subtypes indicating the degree of differentiation of the keratinocyte [16]. In response to injury, keratinocytes in the adjacent skin, as well as those in the supra-basal layer, start to express keratins 6 and 16, demonstrating a more activated phenotype, which then reverts to normal when the wound has been closed [16]. In addition, cells at the leading edge of the wound deposit an extracellular matrix protein (laminin-332), which mediates the keratinocyte migration and anchors cells to the basement membrane [9, 16]. Keratinocytes in chronic wounds, such as diabetic and venous ulcers, are not able to complete these key steps in re-epithelialisation. Whilst they have an activated phenotype and are highly proliferative, they are not well differentiated [16, 17]. They have impaired ability to migrate, which is thought to be because of decreased production of laminin-332 [9]. They also have decreased expression of the growth factors VEGF and TGF-α, although they show increased expression of PDGF when compared with keratinocytes in healthy wounds [9]. Overall, this imbalance in gene expression results in the disorganised hyperkeratosis observed at the non-advancing edges of chronic wounds.

1.4.2 Immune Cells and Inflammatory Mediators

1.4.2.1 Neutrophils

The chemoattractants released by platelet degranulation during haemostasis mean that neutrophils are recruited to the wound early and are required for the control of pathogens at the site of injury. Once activated, the neutrophils adhere to the endothelium of the blood vessels at the wound site, and move into the wound by transmigration through an intracellular junction and then through the extracellular matrix. In order to do this, and also to phagocytose bacteria and damaged extracellular matrix, neutrophils have numerous enzymes contained in cytoplasmic granules and secretory vesicles [18]. These include proteases, such as elastase and cathepsin B, D, and G, and antimicrobials, such as myeloperoxidase and lysozyme [18].

Neutrophils also release a number of cytokines, including interleukin (IL)-1, IL-6, and TNF-α, antimicrobial substances, for example reactive oxygen species (ROS), and growth factors [19]. They are important in the recruitment of other immune cells, such as monocytes, and also in promoting proliferation of keratinocytes, fibroblasts, and endothelial cells.

However, whilst neutrophil activity is essential, it must be carefully regulated and uncontrolled activity is detrimental. Excessive numbers of neutrophils have been observed in non-healing wounds and this results in a pro-inflammatory environment. Overproduction of ROS causes damage to the extracellular matrix, increases matrix metalloproteinase

(MMP) activation, and leads to early cell senescence [19]. Levels of MMPs and other neutrophil-produced proteases such as neutrophil elastase are increased in chronic wound fluid compared with acute wound fluid [20]. Increased protease activity breaks down growth factors in the wound environment, reducing their effects. Adhesion molecules such as fibronectin are also broken down, impairing the cell adhesion that is needed for wound closure [21].

1.4.2.2 Macrophages

Monocytes arrive in the wound 5–6 h after injury, and differentiate into macrophages. In addition to monocytes recruited from the circulation by chemokines, there is a population of resident tissue macrophages that proliferate in response to injury. Macrophages are important in all stages of wound healing, and their actions and phenotype change as wound healing progresses [22]. In the inflammatory stage, activated macrophages clear damaged tissue and control pathogens through phagocytosis and antigen presentation to T cells. They secrete a number of pro-inflammatory cytokines and growth factors IL-1, FGF, VEGF, and PDGF [19]. These pro-inflammatory 'M1' macrophages undergo apoptosis a few days after injury. However, a second population of 'M2' macrophages survive to the proliferative phase [19]. Their phenotype changes, and they have a role in the stimulation of keratinocytes, fibroblasts, and endothelial cells to re-epithelialise the defect, deposit new extracellular matrix, and carry out neovascularisation [22]. During this phase, macrophages are important in the production of TGF-β and VEGF, the effects of which are discussed in more detail in Section 1.4.4.

In chronic wounds, macrophage numbers are increased [23]; however, the cells present are thought to be dysfunctional. The switch from the pro-inflammatory 'M1' phenotype to the anti-inflammatory 'M2' phenotype is impaired [19]. Studies in diabetic mouse models have shown that if macrophages do not undergo phenotypic conversion there is a reduction in key growth factors (TGF-β, VEGF, IGF-1), and therefore failure to move into the proliferative phase of wound healing [24]. Additionally, the macrophages in these chronic wounds have a reduced phagocytic capacity, and the resulting build-up of debris and pathogenic material perpetuates the pro-inflammatory state in the wound [25].

1.4.2.3 Tumour Necrosis Factor Alpha

TNF-α is secreted by many cell types in the wound environment, including keratinocytes, fibroblasts, vascular endothelial cells, and inflammatory cells (neutrophils and macrophages). TNF-α stimulates its own release, as well as the production of IL-1, and upregulates the production of MMPs whilst downregulating the production of tissue inhibitors of MMPs (TIMPs) by macrophages, keratinocytes, and fibroblasts. In low concentrations for a short period, this is beneficial, as wound healing is enhanced by the removal of damaged tissue and the stimulation of inflammatory cells and resulting growth factor production [10]. However, prolonged and increased TNF-α secretion delays wound healing as the overall result of sustained TNF-α signalling is the degradation of the extracellular matrix, as well as a number of growth factors and their receptors [10].

Whilst the release of TNF-α is part of the normal cytokine response to injury, the usual pattern in an acute wound is that the increase in TNF-α is limited and transient. In chronic

wounds, the pro-inflammatory cytokine cascade is prolonged and amplified because of the persistence of noxious stimuli [10]. Studies of wound fluid from chronic wounds have found markedly elevated levels of TNF-α compared with healthy surgical wounds [26].

1.4.2.4 Interleukin 1

In the skin IL-1 is manufactured and stored in keratinocytes, ready for release when injury occurs. It is therefore present from the very beginning of the haemostasis and inflammatory stages of wound healing. Levels are further increased by the release of IL-1 from other inflammatory cells, such as macrophages, once they are activated at the site of injury. IL-1 is a chemokine for neutrophils, which are required in injury to remove pathogens. Chronic wounds have increased levels of IL-1 [26] and, in many cases, this is at least partly in response to the presence of bacteria. These wounds also have elevated levels of proteolytic enzymes, such as collagenases, gelatinases, and stromelysins, whose production is induced by IL-1 and TNF-α [26].

1.4.3 Reactive Oxygen Species

ROS are released from endothelial cells in response to TNF, PDGF, and thrombin, from fibroblasts in response to IL-1, and also from neutrophils and macrophages [27]. They are essential in oxidative bacterial killing, and enhance the chemotaxis of neutrophils; they are, therefore, important in the prevention of wound infection. ROS production by nicotinamide adenine dinucleotide phosphate hydrogen (NADPH)-linked oxygenase is highly oxygen dependent [27]. However, like most processes in the inflammatory stage of wound healing, regulation is critical, as very high concentrations of ROS are damaging. Antioxidants such as nitric oxide are produced, and reductases are activated to prevent oxidative damage. Again, these processes are dependent on oxygen [27]. The chronic wound environment is often hypoxic, either as a result of systemic or regional disease, for example atherosclerosis or venous hypertension, or as a result of local factors such as infection, or a combination of both. There is often a repetitive cycle of ischaemia and reperfusion, such as when a leg with a poor arterial blood supply is elevated and then dependent. In such circumstances, there is a net build-up of ROS and an increase in inflammation and tissue damage [27].

1.4.4 Growth Factors

1.4.4.1 The Platelet-Derived Growth Factor Family

PDGF is made up of a family of homo- or heterodimeric growth factors, which bind to three different transmembrane tyrosine kinase receptors [28]. PDGF is released by platelet degranulation during the haemostasis stage of wound healing, and is found in high concentrations in wound fluid early after injury [29]. It has a chemotactic effect on neutrophils, monocytes, fibroblasts, and smooth muscle cells [30]. In addition, PDGF stimulates fibroblasts to proliferate and synthesise extracellular matrix components [28]. It promotes angiogenesis in hypoxic conditions, and in vivo experiments have demonstrated that PDGF increases pericyte and smooth muscle cell recruitment to the new capillaries, increasing structural integrity [30].

PDGF levels are decreased in chronic wounds [11]. It is thought that this is due to increased MMP and neutrophil elastase activity in chronic wounds, as PDGF degradation can be reversed if these enzymes are inhibited [30].

There has been interest in PDGF as a potential treatment in non-healing wounds, and it is the only growth factor approved by the United States Food and Drug Administration available for clinical use. The results of pre-clinical experiments were promising, but there has been only limited success in translational clinical trials. A systematic review in 2013 found a small benefit over standard care in achieving complete wound closure; however, the authors commented that the quality of the clinical trials reviewed meant that the strength of the evidence was low [31].

1.4.4.2 The Epidermal Growth Factor Family

This family includes a number of members which are important in wound healing: EGF, heparin-binding EGF (HB-EGF), and TGF-α [30].

EGF is secreted by platelets, macrophages, and fibroblasts. It is a potent chemotactic for a number of cell types, including keratinocytes. HB-EGF and TGF-α are produced by keratinocytes and macrophages [32]. These growth factors all activate the EGF receptor (EGFR), a tyrosine kinase transmembrane protein found throughout the dermis although most prominent in the basal layer [30, 33]. HB-EGF and TGF-α have been found in high concentrations in wound fluid. Schultz et al. [33] found that the fluid collected from the drains of mastectomy wounds stimulated fibroblasts in vitro. It was rich in peptide growth factors, including TGF-α, IGF-I, and TGF-β. In comparison, fluid collected from chronic wounds had low levels of growth factors, and did not stimulate fibroblasts in vitro. This inhibition of mitogenesis was reversible when acute wound fluid was added [33]. Other groups have also found that fluid from chronic wounds not only inhibits fibroblast proliferation but also decreases cell viability in vitro [34].

EGFR expression is upregulated in the proliferative phase of acute wound healing, but subsequently declines [28]. Re-epithelialisation is significantly impaired in EGFR knock-out mice compared with similar injuries in wild-type mice [35]. Alterations in the expression of EGFR in chronic wounds has been demonstrated. Brem et al. [36] used histology, gene expression profiling, and in vitro migration assays to analyse skin biopsies from the edge of non-healing venous ulcers. They used healthy skin adjacent to the wound edge from the same patients as a comparison, as well as 'normal skin' biopsies as a control. They noted that wound edge biopsies had a 'distinct pathogenic morphology', with a hyperproliferative epidermis, dermal fibrosis, and increased pro-collagen synthesis. When cultured in vitro, fibroblasts from these biopsies demonstrated impaired migration. The gene expression profile of the wound fibroblasts was reproducibly altered, and immunohistochemistry demonstrated reduced EGFR expression. The EGFR that was present was predominantly cytoplasmic. In fibroblasts from adjacent skin there was increased EGFR expression, and the receptor was present at the cell surface as well as in the cytoplasm. In the control samples, EGFR was only expressed at the cell membrane and expression was reduced compared with the adjacent skin samples.

These studies suggest that the EGF family and its receptor play an important role in wound healing by driving the expansion of the keratinocyte population in the wound, promoting both proliferation of existing cells and migration of cells from the

surrounding healthy skin. However this process must be correctly regulated for successful re-epithelialisation.

1.4.4.3 The Fibroblast Growth Factor Family

The FGF family is a group of structurally related polypeptides which act at tyrosine kinase transmembrane protein receptors. The four FGF receptors bind the different FGFs with variable affinity. FGFs are generally mitogenic, stimulating a broad range of cell types, including fibroblasts and keratinocytes to proliferate, but also to migrate or differentiate in some cases. [28].

FGFs have been found in wound fluid early after injury [12, 29]. Studies have also demonstrated increased expression of FGFs during wound healing, and that reduction in the expression of FGFs increases the likelihood of wound healing disorder [37]. An in vivo study of diabetic mice, which demonstrate impaired wound healing, found that FGFs were expressed at lower levels and for less time than in wild-type mice with similar injuries [37].

FGFs have been investigated as a potential treatment for chronic wounds, with studies carried out in patients with pressure ulcers [38] and diabetic foot ulcers [39]. However, the results of clinical trials have not supported the introduction of FGF supplements into clinical practice.

1.4.4.4 The Vascular Endothelial Growth Factor Family

The VEGF family includes six subtypes that bind to three transmembrane tyrosine kinase receptors. VEGF-A and its receptors VEGF receptors 1 and 2 (VEGFR-1 and VEGFR-2) have been the most extensively studied in greater detail and found to be important in angiogenesis and vasculogenesis [28]. VEGF is produced in response to hypoxia, and also in response to several other growth factors, including TGF-β, FGF, and PDGF [38]. Animal studies in rats and guinea pigs have shown that VEGF expression is significantly elevated in keratinocytes at the edge of an acute wound, and remains high in the keratinocytes that move to close the defect [40]. Platelets, macrophages, and keratinocytes also secrete VEGF during wound healing [38].

When activated, the VEGF receptors trigger multiple events: vascular permeability is increased, enabling extravasation of neutrophils and monocytes, MMP-1 and MMP-2 are induced, activating plasminogen and breaking down the basement membrane, and endothelial migration is stimulated [38, 40]. All of these processes are essential for angiogenesis. VEGF acts on smooth muscle cells, increasing the production of MMPs and stimulating migration and proliferation [41]. The same receptor pathway is present in monocytes, stimulating migration and activating the cells to produce tissue factors [41]. Other effects include fibroblast proliferation and keratinocyte motility [38]. VEGF, along with nitric oxide and MMP-9, is thought to be important in endothelial cell progenitor migration from the bone marrow [9]. These progenitor cells are essential for wound healing, although the exact mechanisms of recruitment and homing to the wound site are not yet clear [9].

Angiogenesis is impaired in chronic wounds [42] and it is therefore not surprising that VEGF expression and processing have also been found to be altered in chronic

wounds. Soluble VEGFR-1 (sVEGFR-1) is an endogenous inhibitor of VEGF. A high level of sVEGFR-1 in a wound is a poor prognostic sign [43]. It is thought that sVEGFR-1 acts as a decoy receptor, mopping up the VEGF in the wound and preventing it from activating its target pathways. Expression of sVEGFR-1 is increased in chronic wounds [42]. Furthermore, a study of biopsies from chronic venous ulcers found that, although VEGF and VEGFR expression was elevated compared with normal uninjured tissue, it was not as high as in psoriatic lesions, which were used as a positive control [44]. Recombinant VEGF was then incubated with fluid from the chronic wounds, and fluid from acute wounds. Western blotting demonstrated that in chronic wound fluid the VEGF was broken down, whereas in acute wound fluid it remained stable [44]. It is suggested that this increased proteolytic activity in a chronic wound is the reason why, despite the increased expression of VEGF, its beneficial effects are not observed [42]. It is also likely to be the reason that treatment with exogenous VEGF does not improve wound healing.

1.4.4.5 The Transforming Growth Factor Family

Whilst there are over 30 members of this growth factor family, only a few of them have been implicated in wound healing: TGF-β1, -β2, and -β3 are synthesised by macrophages, platelets, keratinocytes, and fibroblasts, and activins βA and βB are expressed by fibroblasts, endothelial cells, and keratinocytes [38]. TGF-β1 is generated by platelets in an active form, but all the other members of the family are produced as precursors, in an inactive form [38, 45]. They are sequestered bound to proteins linked to extracellular matrix components, and therefore require enzymatic activation by proteases [45].

TGF-β1, -β2, and -β3 are important in wound healing for the recruitment and migration of inflammatory cells, fibroblasts, and keratinocytes. More specifically, TGF-β1 and -β2 induce differentiation of fibroblasts to myofibroblasts, thus increasing extracellular matrix deposition and prompting wound contraction and scar formation [30]. However, the concentration of TGF-β1 affects its action on cells, with low levels promoting endothelial proliferation and migration and high levels increasing extracellular matrix deposition by stimulating collagen deposition and inhibiting MMPs through the increased expression of TIMPs [22, 45].

Levels of the TGFs are decreased in chronic wounds, and as with other growth factors this is likely to be a result of excessive protease activity in the wound bed. In addition, the action of TGFs is impaired in chronic wounds by a decrease in receptor expression on target cells [30].

1.4.4.6 Insulin-Like Growth Factor

There are two types of IGF: IGF-1 and IGF-2. They are released by platelet degranulation, and also synthesised by fibroblasts. The IGF receptor, a transmembrane tyrosine kinase receptor, stimulates mitogenesis and increases survival in a number of cell types [28]. It is thought that IGF is a factor in the aetiology of chronic wounds associated with diabetes and glucocorticoid treatment because the expression of IGFs and their receptors is abnormal in these conditions [28]. Immunohistochemistry comparing skin from a diabetic foot

ulcer with uninjured diabetic skin and non-diabetic skin found that, whilst the expression of IGF-2 was comparable in all samples, the expression of IGF-1 was markedly different. In non-diabetic skin, IGF-1 was widely expressed throughout the epidermis, whilst in uninjured diabetic skin it was only found in the stratum granulosum and spinosum, and in ulcerated diabetic skin it was absent [46]. Fibroblasts from the tissue samples from patients with diabetes also lacked IGF-1 [46].

1.4.5 The Role of Matrix Metalloproteinases

The MMPs are a group of proteases, a number of which have a role in wound healing. MMP-1 and MMP-8 are collagenases. Their substrates include collagen I, the predominant collagen of the skin, and collagen III, the collagen initially laid down to close a wound defect. MMP-1 is expressed on the first day after injury, but then gradually decreases and MMP-8 becomes the main collagenase in the healing wound [47]. MMP-2 and MMP-9 are gelatinases, and their substrates include gelatin, type I collagen, and type IV collagen, which are found in the basement membrane. They are expressed by keratinocytes and enable cell migration [47]. MMP-3 and MMP-10 are stromelysins, and can break down collagens, as well as non-collagenous matrix macromolecules including fibronectin, elastin, and gelatin [47]. These activities are important for successful wound healing. Breakdown of the basement membrane is required to allow migration of monocytes and neutrophils from the vasculature into the tissue. Damaged extracellular matrix must be broken down and removed. The initial granulation tissue laid down is disorganised and not as strong as native tissue, and must therefore be remodelled once the epithelial defect has been closed.

In healthy tissue there is minimal expression of MMPs, but if remodelling is required they can be rapidly upregulated in a number of different cell types, including keratinocytes, fibroblasts, endothelial cells, monocytes, lymphocytes, and macrophages. They are produced in response to cytokines and growth factors, including EGF, FGF, VEGF, PDGF, TNF-α, TGF-β, and some interleukins. MMPs are initially produced in an inactive form (pro-MMPs), and subsequently activated by serine proteases or other MMPs. Neutrophil elastase is one such activating enzyme [48].

MMP activity must be tightly controlled in order to enable repair whilst avoiding tissue damage. In addition to the pathways described above, which promote gene expression and MMP activation, there are also inhibitors of both the MMPs themselves and the enzymes which activate the pro-MMPs. TIMPs are proteins produced by cells in the wound, and are also found in serum. There are three forms of TIMP, all of which have been shown to be active in wound healing, and specifically in the control of cell migration and extracellular matrix remodelling [47]. They bind and inhibit activated MMPs.

The normal control of MMP activity is dysfunctional in chronic wounds. However, it is a complex situation. Increased MMP activity has been proposed as a causative factor in chronic wounds. Elevated levels of MMP activity have been found in wound fluid from venous leg ulcers [20, 49], diabetic foot ulcers [20], and pressure ulcers [50], when compared with that from acute wounds. In these studies MMP-2 and MMP-9 were specifically implicated. However it is also suggested that inadequate MMP activity can be a problem, with another study suggesting that overexpression of TIMP-1 and -2 and the resultant decreased levels of active MMP-1 and MMP-2 caused the defective extracellular matrix reorganisation and failure of healing in chronic leg wounds [51].

1.5 How a Chronic Wound Develops: Extrinsic Factors

There are a number of extrinsic factors that have been shown to predispose a person to developing a chronic wound. They are summarised in Figure 1.3. They share a common overall effect, which is that inflammation is promoted in the wound site, and this leads to impaired function of the wound cells and failure of the normal healing processes.

1.5.1 Infection

The duration of a wound as well as the resulting morbidity and mortality are all increased by the presence of infection. The persistently high bacterial counts present in colonised wounds are a key driver of the inflammatory response, and the bacteria, the toxins they produce, and the inflammatory cells activated by them are all detrimental to the wound environment. There is an increased concentration of proteases, which break down the extracellular matrix, as well as key growth factors and their receptors in the wound bed [9]. Some of these are released by bacteria, such as the zinc metalloproteinase, elastase, produced by *Pseudomonas aeruginosa* [52]. The release of host MMPs and leukocyte-derived proteases, such as neutrophil elastase, is also increased. Whilst invasive infection is a problem, often the bacteria are localised in a biofilm. It is thought that these secreted polymer matrices enable bacteria to evade host immune defences and enhance production of virulence factors, significantly delaying wound re-epithelialisation in animal models [53].

1.5.2 Nutrition

1.5.2.1 Hyperglycaemia

Diabetes is known to be a risk factor for chronic wounds. The metabolic syndrome that is often present in patients with diabetes means that there is a greater likelihood

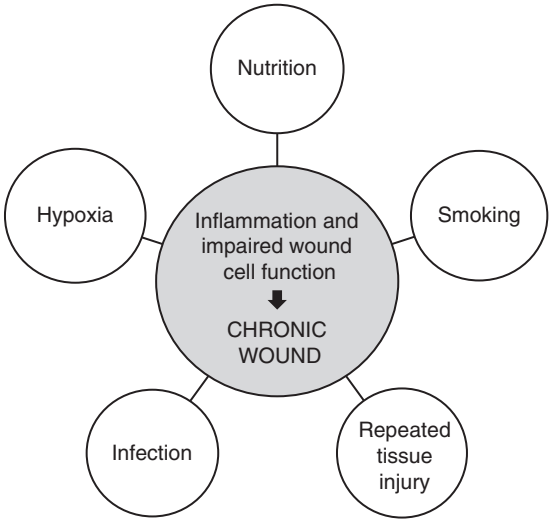

Figure 1.3 *Extrinsic factors contributing to the development of a chronic wound.*

of atherosclerosis and small vessel disease, resulting in tissue ischaemia, as well as an impaired immune response to infection. However, the hyperglycaemic state also has specific effects in the wound environment which are detrimental to healing and the hyperglycaemia causes glycation of proteins. Glycated collagen is cross-linked, insoluble, and stiffer than normal, which adversely affects fibril assembly. Binding with proteoglycans is also affected, which affects matrix assembly and stability [54]. Proteoglycan binding is essential for cell–collagen interactions with keratinocytes and fibroblasts, and adhesion and migration of these cell types is impaired when collagen is glycated [54]. Hyperglycaemia also stimulates MMP production by fibroblasts, macrophages, and endothelial cells, and the extracellular matrix is broken down if MMP concentrations are too high [55].

1.5.2.2 Malnutrition

The increased activity at the site of injury means that there is an increased requirement for macro- and micronutrients for successful wound healing. Proteins, fats, and carbohydrates are all required, as well as vitamins and minerals.

A common problem in the elderly population is protein energy malnutrition, where an underconsumption of protein and a calorie deficit result in weight loss, and specifically a decrease in lean body mass. This problem is exacerbated when there is a chronic wound, as protein is mobilised to meet the metabolic demand in the wound, and wound exudate is protein rich. Any decrease in lean body mass will affect wound healing, with impaired immunity and increased risk of infection in losses of 10%, thinned skin and decreased wound closure rates in losses of up to 20%, and complete failure of wound healing and high risk of new wounds once losses of 30% have occurred [56].

Vitamin K is essential in the production of clotting factors, and therefore vital in the haemostasis stage of wound healing. A number of vitamins and minerals, including vitamins A and C, zinc, copper, and manganese, are essential in collagen synthesis [57]. Zinc is also a co-factor in the production of other proteins, and for DNA and RNA polymerase, and is therefore important in fibroblast proliferation [57]. Vitamin A deficiency results in delayed re-epithelialisation.

1.5.2.3 Obesity

Obesity has been linked to a pro-inflammatory state, and it is proposed that this is an underlying reason for the poor wound healing in this group. Mouse and rat models of obesity, through dietary or genetic modifications, have demonstrated delayed wound healing, and reduced wound strength compared with non-obese controls [58]. Inhibiting the systemic inflammatory response in genetically obese mice by treating them with neutralising antibodies against TNF-α and F4/80, a macrophage cell surface protein, increased the healing rate [59].

1.5.3 Tobacco Smoking

The negative effects of tobacco smoking on multiple organ systems are well described, and there are many studies which have found that smoking is detrimental to wound healing [60–63]. Smoking affects the function of many wound cell types, including fibroblasts, neutrophils, and macrophages [64]. A number of reasons for this are proposed.

Cigarette smoke decreases the oxygen concentration in the tissues [65]. A study in current and ex-smokers found that, in the hour after smoking a single cigarette, cutaneous and subcutaneous blood flow, tissue oxygen tension, and tissue glucose concentration were significantly reduced, while tissue lactate concentration significantly increased [61]. Arterial occlusion in the limb using a blood pressure cuff caused a more pronounced effect with the same pattern of results. The effects of hypoxia are discussed further in Section 1.5.4. Cigarette smoke contains carbon monoxide, nicotine, and hydrogen cyanide, all of which affect oxygen delivery to cells [64]. Other chemicals in cigarette smoke act as oxidants and are thought to inhibit innate immunity, for example by affecting the ability of macrophages to detect bacteria and inhibiting cytokine release in the presence of bacteria [66]. Smoking inhibits the release of ROS from neutrophils and macrophages, reducing their oxidative killing of bacteria [67]. The release of proteases (MMPs and neutrophil elastase) from neutrophils is increased, whilst the production of TIMPs remains the same, resulting in extracellular matrix destruction [67]. Overall, there is an increased propensity towards chronic infection, inflammation, and failure to heal.

1.5.4 Hypoxia and Ischaemia–Reperfusion Injury

The initial cellular activity in the early phases of wound healing is triggered by the hypoxic environment that develops where there has been vascular injury and then vasoconstriction. However, following this, oxygen is an essential requirement for wound healing, and demand is increased by the raised metabolic activity at the site of injury.

Hypoxia is detrimental to cellular activity. As already discussed in Section 1.4.3, the ability of neutrophils and macrophages to move to the wound site and carry out oxidative killing is impaired when tissue oxygen concentrations are low. In addition to dysfunction in the inflammatory phase of healing, hypoxia also affects the ability of cells to proliferate and synthesise extracellular matrix components [27]. In addition to in vitro and animal experiments which suggested that the production of collagen by fibroblasts is dependent on tissue oxygen tension, Jonsson et al. [68] found that, in postoperative surgical wounds, collagen deposition was proportional to wound oxygen tension.

Ischaemia–reperfusion injury is thought to be a factor in the aetiology of many chronic wounds, including arterial and venous leg ulcers and pressure ulcers. It occurs when the delivery of oxygen to the wound bed is intermittently impaired, for example when there is weight bearing on a pressure ulcer or when a leg with impaired circulation is dependent and then elevated. When the tissue is ischaemic, an abnormal inflammatory environment develops, and then when reperfusion occurs there is additional influx of inflammatory cells and exudate and a resulting increase in proteases and ROS, compounding the damage to the tissue [27]. An experimental rat model of pressure ulceration found that ischaemia–reperfusion from repeated pressure cycles was more damaging than a prolonged period of ischaemia [69].

1.6 Concluding Remarks

Regardless of the aetiology, an acute wound must progress through a series of overlapping stages in order to achieve healing. Numerous cell types are involved, and must be carefully orchestrated. The important growth factors have been briefly discussed in this chapter. Inflammation and degradation of extracellular matrix proteins are essential to clear

pathogens and debris, and to enable neovascularisation and migration of cells into the wound. However, these potentially destructive processes must be controlled, and balanced with constructive actions such as the deposition of new matrix proteins and the proliferation of cell populations. Disruption of this complex set of interactions will result in failure of the wound healing process and the development of chronic (non-healing) wounds.

Chronic wounds represent a huge unmet clinical need, resulting in significant morbidity and mortality, and a burden on healthcare resources. Understanding the molecular and cellular processes at play in the wound environment is important in our quest to develop better treatments for chronic wounds. As described above, many changes in cell phenotype have been observed in non-healing wounds, leading to altered behaviour, which affects the synthesis of growth factors, enzymes, and matrix proteins, with chronic infection and a persistent inflammatory response frequently observed. In order to be able to convert a chronic wound into a healthy healing wound, the underlying mechanisms of both situations must be understood and these processes optimised in the chronic wound. The complexity of the wound environment means that this remains a challenging field.

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