The duplicitous nature of inflammation in wound repair

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Abstract

Skin plays a key role in protecting the body from the onslaught of pathogens and toxins we meet during our lifetime; thus, out of necessity, we have developed a rapid repair mechanism that quickly plugs any holes in this vital organ. Upon injury, a series of highly coordinated overlapping events, that include inflammatory, proliferation and maturation phases, result in the hasty closure of the wound and restoration of skin integrity. Over the past decade it has become clear that a number of immune cells that regulate the inflammatory phase, whilst important for removal of invading pathogens, are not necessary for repair and in fact may be responsible for the subsequent scar formation that seems to have resulted from having such a rapid repair process. The magnitude and length of inflammation in the wound not only appears to dictate the extent of scar formation but also in some cases may even prevent wound closure. In this review we will explore the two sides of inflammation in wound healing and review current and future drug therapies that target inflammation to modulate the healing outcome.

Introduction

Evolution has provided us with a complex and highly dynamic series of events to rapidly close a wound and prevent infection after injury. The repair process can be broadly divided into inflammatory, proliferative and remodelling phases (Figure 1) that involve numerous different cell types, some from the local area, while others are recruited upon injury [1-3]. These processes ultimately lead to the elimination of invading organisms, removal of damaged cells and tissue, and re-establishment of the skin barrier. In an ideal world, repair would result in regeneration of the original tissue with structural, functional and aesthetic attributes similar to that of uninjured skin.

The downside of having such a rapid repair process is that structural integrity is maintained by the replacement of damaged tissue with fibrotic material, leading to scarring [4, 5]. There are a few rare exceptions that do not result in scarring, including tattoos, superficial scratches and foetal skin wound healing. Additionally, certain tissues have reduced levels of scarring and repair themselves more rapidly such as oral mucosa wounds [6-8]. One of the main features of both foetal and oral mucosa wound healing is their limited inflammatory response during the repair process [6]. At the other end of the scale are wounds that heal slowly with poor dermal quality, such as diabetic ulcers, where a robust inflammatory response can play a role in prolonging healing time [9]. In this article we will explore the role of inflammation in cutaneous repair, looking at its many functions, mostly beneficial but some potentially deleterious, and review current and future drug therapies that target inflammation to modulate the healing outcome.

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The inflammatory phase of wound healing

Upon injury, platelets are the first blood cells on the scene; they are activated by binding to the collagen exposed when the blood vessel lining is damaged, leading to rapid plugging of the wound with a fibrin-rich clot to prevent blood loss. Platelets secrete biologically active proteins that bind to the fibrin mesh and to the extracellular matrix (ECM), creating chemotatic gradients that trigger the inflammatory phase of repair by recruiting immune cells to the wound [9]. Neurophils are the first nucleated immune cell to infiltrate a wound, acting as a first line of defence by decontaminating the wound [10]. These cells phagocytose foreign material and infectious agents and secrete anti-microbial substances such as reactive oxygen species, cationic peptides and proteases (Figure 2) [10]. Neutrophils also secrete enzymes, such as matrix metalloproteinases (MMPs), which begin debriding devitalised tissue [10]. Usually neutrophil infiltration ceases

after a few days and the act of phagocytosis results in the neutrophil committing suicide by apoptosis.

Around 48 hours after the initial injury, monocytes are recruited via the numerous chemoattractants, including growth factors, cytokines and chemokines, produced by platelets, neutrophils, keratinocytes and fibroblast at the site of injury. In the wound, monocytes differentiate into macrophages and are then an abundant and active component throughout the repair process, lingering long after the wound has closed.

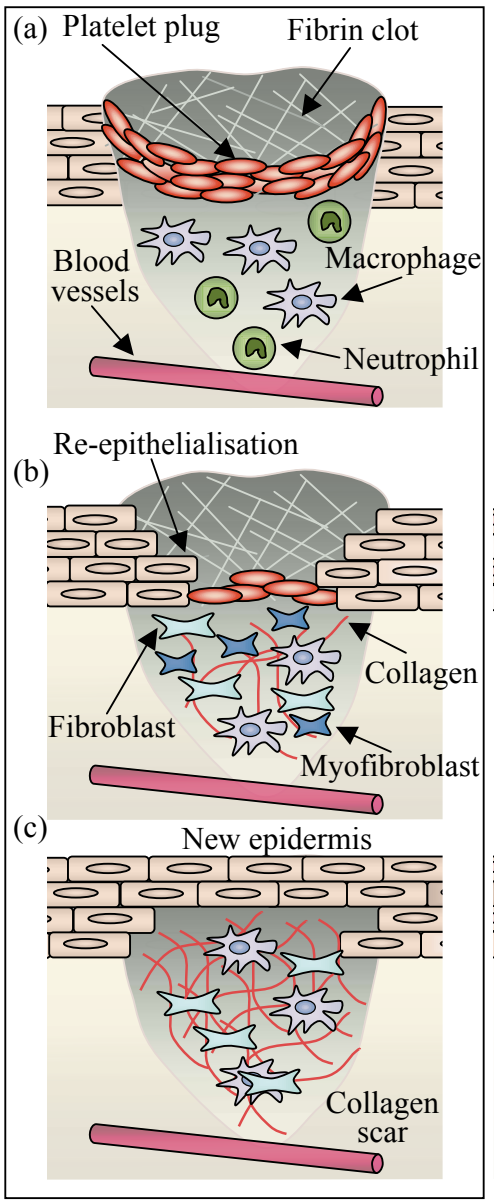


Figure 1. The three phases of a typical wound healing response.

(a) Inflammation – a fibrin clot forms and platelets plug the wound; neutrophils then macrophages migrate into the wound and are responsible for bacterial destruction and removal of foreign material and cell debris.

(b) Proliferation – mediators secreted by macrophages and surrounding cells initiate proliferation and migration of keratinocytes and fibroblasts into the wound; collagen deposition and contraction.

(c) Remodelling – matrix remodelling by macrophages, fibroblasts, endothelial and epithelial cells.

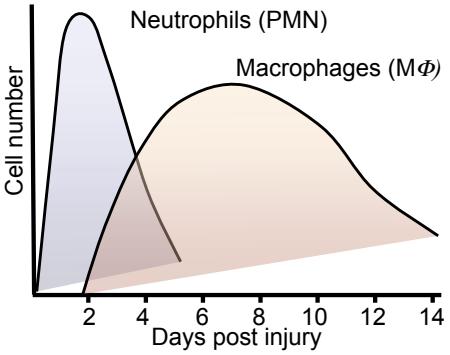
During the repair process, macrophages are thought to play a pivotal role in fibrosis and scarring [9, 11]. Within the wound, macrophages clear the matrix and cell debris, including fibrin and the spent neutrophils. They also secrete a variety of cytokines, growth factors and mediators of inflammation that can coordinate different cell actions, such as fibroblasts proliferation and angiogenesis, during wound closure (Figure 2). Successful repair entails resolution of the inflammatory response. The mechanisms that deactivate the inflammatory response within a wound are not so well understood. Macrophages are capable of switching off their own pro-inflammatory response by secreting anti-inflammatory mediators, such as IL-10 and soluble receptors, that sequester pro-inflammatory molecules.

Immune cells dictate the quality and speed of tissue repair

The inevitable scar formation seen in adult skin following wounding is remarkably absent in the developing embryo; wounds made in the first one third to one half of gestation heal perfectly without scarring [12]. Notably, their repair is rapid and leads to regeneration of the normal tissue structure, with the deposition of large bundles of ECM in the basket-wave orientation found in normal skin. Adult wound repair replaces damaged skin with the deposition of small parallel bundles of ECM that form scar tissue.

Although many differences between adults and embryo wound healing exist, the majority have been shown to be irrelevant to scar formation. For instance, neither the sterile uterus environment, nor amniotic fluid, is responsible for scarless healing seen in the embryo [13]. This was nicely demonstrated using young marsupials who, in the first 9 days post birth, heal without scarring even though they are frequently exposed to maternal urine and faeces while in the pouch [13]. Thus, the womb environment itself is not the key to scarless healing that occurs in the foetus.

One of the major differences between adult wound healing and foetal scarless repair is the extent of inflammation found within the wound [14]. In the foetus, where the immune system is still developing, wounds have strikingly reduced levels of immune cells, such as macrophages, that are less activated and exit the wound much faster than those found in adult wounds [14, 15]. Even at stages of development when macrophages have commenced patrolling tissues, they are not generally recruited to wounds until after wound closure [15]. Not surprisingly, as these cells are



Cell type	Secrete	Functions
PMN	TNFα, IL-1β, IL-6 VEGF, IL-8 Reactive oxygen species, proteases,cationic peptides, eicosanoids	Pro-inflammatory Stimulates repair Anti-microbial
MΦ	TNFα, IL-1β, IL-6 IL-10, TGFβ-1 PDGF VEGF, bFGF, TGFα TGFβ-3	Pro-inflammatory Anti-inflammatory Chemoattractant activates MΦ Stimulates repair;angiogenesis and fibroplasis

Figure 2. Relative numbers of inflammatory neutrophils and macrophages at the site of injury during wound repair with their secreted products and function. The graph indicates the relative number of neutrophils and macrophages in a wound over a typical wounding response time course. The mediators these inflammatory cells secrete and their functions are listed in the table.

the major producers of growth factors in the adult wound, their reduction in numbers in the embryo wound results in lower growth factor levels for shorter periods of time [16-18]. This lack of immune cells persists until relatively late in development when macrophage attendance at the wound coincides with scar formation [15, 16]. Likewise, in the pouch, marsupial scar formation begins around Day 9 when inflammation becomes more prominent [13].

While this is interesting, in itself it does not prove that inflammation during repair is intimately linked to scar formation. To test this hypothesis, inflammation has artificially been induced in foetal wounds either by injecting a potent chemoattractant or by the presence of bacteria in the wound [19, 20]. The foetus is capable of mounting an acute inflammatory response to both of these stimuli, which then induces an adult-like healing response and scar formation [19, 20]. So it appears inflammation may itself lead to the abnormal deposition of collagen and scar formation. Even after birth, a number of exceptional sites in the body exist, such as the mouth, that fail to trigger a standard inflammatory response and have low neutrophil and macrophage infiltration after injury and reduced levels of scarring [7, 8].

A series of classic of experiments in the 1970s endeavoured to directly test the role of the distinct immune cell populations in wound healing using antisera to deplete individual cell types [21, 22]. Under sterile conditions, depletion of macrophages by antisera in combination with steroids resulted in failure to debride the wound and extended wound closure time, whereas antisera depletion of neutrophils had little effect on wound healing [21, 22]. Subsequently, results from these early studies have now been challenged and their findings show no one immune cell type is essential for wound healing. Platelets and mast cells are both individually dispensable to the repair process and their depletion has little effect on the proliferative effects of wound closure, angiogenesis and collagen synthesis [23, 24]. More recent studies, again using anti-neutrophil antisera, confirm neutrophils are not essential to the repair process and suggest neutrophils may actually retard repair since wound closure was greatly accelerated in these mice [25]. Mice (PU.1-knockout mice) that lack macrophages and neutrophils have been shown to have improved rates of wound re-epithelialisation [11] and, more importantly, a lack of fibrosis and reduced scarring compared to wild type mice [11]. So it appears overall that inflammation may play a major role in scar formation, and that removal of any one type of immune cell does not hamper healing; in fact, neutrophils and macrophages are probably responsible for at least initiating fibrosis and scar formation [11, 25].

Inflammation and impaired healing

While most wounds heal without difficulty, there are some instances where the body's natural healing process is deregulated and wounds fail to progress through the typical orderly sequence of repair in a timely fashion. Disruption of one or more of the healing stages can result in prolonged and incomplete repair, with lack of restoration of integrity. Non-healing wounds are a significant problem for healthcare systems all over the world; it is estimated that in Australia alone 270,000 people suffer from chronic wounds. These wounds can cause significant pain and suffering, loss of independence and often interfere with quality of life. A variety of chronic wounds exist; some are associated with complications from diabetes and circulatory problems such

as venous and diabetic ulcers, while others can result from immobility, traumatic injury such as deep burns or non-healing surgical incisions.

Often the delay in tissue repair results from a disruption in the inflammatory phase of repair, with many different factors contributing to poor healing such as wound infection, foreign objects such as sutures, or the presence of debris and necrotic tissue. Non-healing wounds have some distinct characteristics. They frequently have high bacterial load in combination with growth factor, inflammatory mediator and proteolytic enzyme imbalances that favour tissue degradation over repair. Neutrophils and macrophages are abundant in these wounds and secrete many of the bioactive substances that in high concentrations exacerbate tissue damage [10]. Excess secretion of proteases, such as the MMPs, capable of degrading essentially all extracellular components and basement membrane proteins, can lead to substantial tissue damage [26, 27].

Re-epithelialisation requires cells at the wound margin to loosen their cell-ECM and cell-cell interactions in order to migrate across the wound and MMPs function in part to facilitate this local ECM remodelling during repair [27]. However, excess secretion can induce uncontrolled tissue degradation, including new granulation tissue and growth factors, delaying collagen deposition, so impairing the repair process. These enzymes and others in the wound activate additional enzymes, release growth factors from the cell surface or ECM, cleave cell adhesion molecules from the plasma membrane, and convert wound cytokines into an active or inactive form, contributing to the non-healing phenotype. Reactive oxygen species released by these cells to fend off infection also inhibits cell migration and proliferation and can cause tissue damage, again exacerbating the problem [28]. The continued production of pro-inflammatory cytokines and chemokines further attract and activate additional inflammatory cells, perpetuating the non-healing condition. The necrotic tissue itself also impairs healing as it provides a rich growth environment for bacteria, increasing the chance of infection and so increasing inflammation in the wound. Endotoxins from the devitalised tissue also inhibit fibroblast and keratinocyte migration into the wound. So, for non-healing wounds, increased numbers of immune cells, their secreted bioactive substances and inflammation can be inhibitory to repair, greatly prolonging healing time.

Dampening down inflammation therapeutically

Unravelling the mechanisms that generate rapid, perfect skin repair in the foetus has been one of the major research goals in wound healing in recent years. Research is rapidly providing clues that may allow the subtle harnessing of inflammation to improve the quality of healing. Discovery of the differential expression of cytokines and growth factors, such as IL-6, IL-10, platelet-derived growth factor (PDGF) and transforming growth factor-beta (TGF-ß), in embryonic and adult wounds, has lead researchers to target altering their levels to recapitulate those found in foetal wounds in an effort to reduce inflammation and make the adult wound environment more foetal-like and improve repair [29-35].

The addition of the pro-inflammatory cytokine IL-6, a key chemoattractant for monocytes and a macrophage activator, leads to scarring in foetal wounds, suggesting that the decreased IL-6 levels found in foetal wounds may help provide an environment conducive to regeneration rather than scarring [33]. Down-regulating the acute increased IL-6 levels at the site of injury thus may improve healing outcomes.

IL-10, a potent anti-inflammatory cytokine, produced by macrophages and to a lesser extent lymphocytes, deactivates macrophages and reduces pro-inflammatory cytokine secretion from macrophages. Mice lacking IL-10 have accelerated re-epithelialisation, with increased levels of macrophages and enhanced contraction in the adult wound tissue [36]. However, foetal wounds in these mice show increased scarring with reduced matrix deposition and biomechanical strength of the wound, suggesting IL-10 levels affect the quality of tissue repair [32]. Over-expression of IL-10 in wild type adult mice wounds leads to a reduction in pro-inflammatory mediators and inflammation, normal collagen deposition and restoration of normal dermal architecture similar to that found in foetal wounds [34]. So it appears that increasing IL-10 levels at the time of wounding may reduce subsequent scaring by providing an environment conducive to regeneration. To this end, Renovo [Manchester, UK] have developed a human recombinant IL-10 (Prevascaris), for use in the prevention and reduction of scarring in skin, which is now in Phase II clinical trials.

Both foetal and adult oral mucosa wounds heal rapidly with little or no scaring and limited inflammation [8, 37]. What they also have in common is lower levels of TGF-ß1 compared with adult dermal wounds, along with a significant increase in the ratio of TGF-ß3 to TGF-ß1 [7, 16, 17, 38]. TGF-ßs are signalling molecules secreted by platelets, fibroblasts and macrophages that play distinct roles during repair. TGF-ß1 is present through all stages of the repair process and promotes immune cell recruitment as well as increasing matrix protein synthesis while decreasing matrix protein degradation leading to fibrotic tissue formation. Increasing levels of TGF-ß1 in the foetal wound results in scaring [37], while addition of TGF-ß1 and TGF-ß2 neutralising antibodies to adult wounds reduces scarring [35, 39]. Exogenous TGF-ß3, when injected into wound margins, reduces monocyte and macrophage infiltration,

fibronectin, collagen I and III deposition, resulting in reduced scarring in adult tissue [39]. Results suggest that it is the ratio of TGF-ß3 to TGF-ß1 that may be important in determining scar formation, with higher levels of TFG-ß3 tipping the balance towards reduced scaring. To this end, Renovo [Manchester UK] are about to begin Phase III trials using human recombinant TFG-ß3 as a prophylactic scar reducer given at the time of surgery. Mannose-6-phosphate (Juvidex), from the same company, inhibits the action of both TGF-ß1 and TGF-ß2 and is also currently in Phase II clinical trials for use in accelerating healing time. Comparison of genes from wounds in 'macrophageless' mice – incapable of raising a standard inflammatory response that repairs skin wounds rapidly with reduced fibrosis – with those from wild-type mice have allowed researchers to distinguish genes not absolutely essential to the repair process (that are associated with inflammation in the wound) from tissue repair genes [40, 41]. These up-regulated inflammation-associated genes identified are thought to contain genes that contribute to the negative side effects of inflammation, including retardation of re-epithelialisation and fibrosis.

One such gene, osteopontin (OPN), has now been identified as a good therapeutic target to improve healing [41]. OPN regulates many diverse cellular roles including immune regulation where it acts as a chemokine-like protein [42] to recruit monocytes and macrophages and regulate cytokine production in macrophages, dendritic cells and T-cells. Reducing levels of OPN in wounds has been shown to accelerate healing and reduce granulation tissue formation and scarring [41]. The same study showed OPN is produced by wound fibroblasts in response to PDGF secreted by macrophages and that blocking PDGF receptor signalling reduces OPN levels [41]. In a separate study, over-expression of PDGF in foetal wounds induces fibrosis [29]. Thus, both OPN and PDGF may be good potential therapeutic targets to improve the rate and quality of healing when given at early time points after healing. Further testing of other genes may provide other good targets for therapeutic intervention.

A number of other proteins have been identified as good targets to influence wound repair, including proteins such as connexin43 [43, 44]. Gap junctions between cells are a major route for cell to cell communication and reduction of one of its major components, connexin43, transiently decreases its levels in epidermal cells at the wound edge along with cell to cell communication. Further, reducing connexin43 in wounds reduces immune cell infiltration to the wound and

leads to enhanced wound healing with a reduced overall area of granulation [44].

p38 mitogen-activated protein kinase (p38MAPK) is a signalling molecule found to influence a whole host of cellular events, including inflammation [45]. Topical p38MAPK inhibition in a burn wound has been shown to reduce inflammation within the wound and prevent the subsequent apoptosis often seen after the initial thermal injury [46]. In this study, the end point was to look at the effects of p38MAPK inhibition on initial inflammation levels and apoptosis in the wounds. It is possible that, with this, reduced inflammation in the presence of inhibitors may lead to improved quality and rates of healing, although this has yet to be tested.

What each of the above treatments have in common is a reduction in immune cell infiltration and inflammation; however, the timing of intervention is crucial for a number of the scar reducing therapies developed. All of the above-mentioned targets have been altered in the initial stages of healing to reduce levels of inflammation at the start of repair to modulate the subsequent scarring. What about dampening inflammation in non-healing wounds where increased inflammation exacerbates the non-healing phenotype? Because infiltration of immune cells into many different tissues, including the skin, contributes to inflammation in a range of diseases, the molecules that regulate this have been widely studied [47]. Anti-migration therapy has had mixed results in other diseases but none have yet been tested in wound repair.

Summary

Inflammation plays both positive and negative roles in cutaneous repair – the level of inflammation can dictate both healing time and quality of repair. Recapitulating the dampened inflammatory response found in foetal and adult mucosal wounds has proved useful in accelerating repair and reduced scaring in mice wound models. Currently there are no prescription drugs that prevent or treat scarring, although a number of the potential therapeutic targets that dampen inflammation and reduce scaring are now in clinical trials and we wait with bated breath for the outcome of these. With the current search for proteins that contribute to the negative effects of inflammation and fibrosis, we will hopefully in the future be able to subtly control inflammation to harness only its beneficial effects. The next challenge will be to determine how we can subtly alter inflammation at later time points to accelerate repair, particularly in non-healing wounds where inflammation hampers healing.

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