Part I

Cellular and clinical aspects of scar formation
Potential cellular and molecular causes of hypertrophic scar formation

Willem M. van der Veer
Monica C.T. Bloemen
Magda M.W. Ulrich
Grietje Molema
Paul P.M. van Zuijlen
Esther Middelkoop
Frank B. Niessen
Abstract

A scar is an expected result of wound healing. However, in some individuals, and particularly in burn victims, the wound healing processes may lead to a fibrotic hypertrophic scar, which is raised, red, inflexible and responsible for serious functional and cosmetic problems. It seems that a wide array of subsequent processes are involved in hypertrophic scar formation, like an affected haemostasis, exaggerated inflammation, prolonged reepithelialization, overabundant extracellular matrix production, augmented neovascularization, atypical extracellular matrix remodeling and reduced apoptosis. Platelets, macrophages, T-lymphocytes, mast cells, Langerhans cells, and keratinocytes are directly and indirectly involved in the activation of fibroblasts, which in turn produce excess extracellular matrix. Following the chronology of normal wound healing, we unravel, clarify and reorganize the complex molecular and cellular key processes that may be responsible for hypertrophic scars. It remains unclear whether these processes are a cause or a consequence of unusual scar tissue formation, but raising evidence exists that immunological responses early following wounding play an important role. Therefore, when developing preventive treatment modalities, one should aim to put the early affected wound healing processes back on track as quickly as possible.
Introduction

When cutaneous integrity is violated, wound healing, including scar formation, is crucial in order to restore this barrier. Excessive blood loss is prevented by the formation of a blood clot, which further acts as a provisional wound matrix that attracts and guides inflammatory cells, endothelial cells, fibroblasts, and keratinocytes. Together they form new blood vessels, produce extracellular matrix (ECM) and migrate from the wound edges to create a layer that covers the surface of the wound. Many fibroblasts transform into myofibroblasts, which are considered to initiate collagen deposition and wound contraction.

Except for shallow (burn) wounds that heal within a few days, most wounds will become a visible scar. However, in some individuals, and particularly in burn victims, the wound healing processes may lead to production of overabundant ECM, resulting in a raised hypertrophic scar. They are easily identified by color mismatch, stiffness, and rough texture. Patients frequently complain about itching and pain, and experience serious functional and cosmetic problems, which are caused by a myriad of complications, including compression, stiffness sensation, loss of joint mobility, and anatomic deformities. These complications may require several surgical corrections, unfortunately not always with satisfying results.

Hypertrophic scars are different from keloids, which also raise above skin level, but proliferate or originate beyond the confines of the original lesion. Most of the available literature on hypertrophic scars and keloids still does not precisely differentiate between both scar types, although several pathological and biochemical differences between hypertrophic scars and keloids suggest that different mechanisms are responsible for their development. Therefore, it is important to clearly differentiate between these two types of scars when trying to unravel the pathogenesis of either of these. In this review, we have tried to focus specifically on hypertrophic scars. Following the chronology of normal wound healing, we will make an effort to clarify and reorganize the complex molecular and cellular mechanisms that may be responsible for a hypertrophic scar.

Affected Haemostasis

Usually, wounding involves injury of blood vessels, which consequently causes outflow of blood. The surface of burn wounds differs from these wounds, as they are characterized by coagulation of the superficial blood vessels, and usually do not tend to bleed excessively. However, after excision of the burned eschar, deep and excessively bleeding wounds are created. The most important mediators of haemostasis are blood vessels, platelets, and fibrin. Within seconds after wounding,
blood vessels constrict, platelets aggregate and the clotting and complement cascade are activated. Together they are responsible for the formation of a haemostatic blood clot, composed of crosslinked fibrin, fibronectin, vitronectin, and trombospondin. This will act as a scaffold for wound repair and a reservoir of cytokines and growth factors\(^5,10\). Fibrin and fibronectin facilitate the migration of dermal fibroblasts and endothelial cells\(^11,12\). In normal wound healing, fibronectin expression decreases within a few days after the wound has closed, but in hypertrophic scars, the fibronectin deposition continues at a high level for months to years, possibly responsible for a higher fibroblast density, as seen in hypertrophic scars\(^13\).

Shortly after the formation of the fibrin clot, endothelial cells initiate degradation of the clot. They produce tissue-type plasminogen activator (tPA), which in turn converts plasminogen into plasmin, the major fibrinolytic protease\(^14\). The by-products of the proteolysis of fibrin also act as chemotactic signals to attract neutrophils and monocytes\(^9\). Inadequate removal of fibrin impedes the normal wound healing processes, and might lead to fibrosis\(^15,16\). For example, fibrin deposits and suppressed fibrinolysis are found in keloids and pulmonary fibrosis\(^16\). Fibrinolysis is controlled by either transforming growth factor (TGF)-\(\beta\)1 which upregulates, or TGF-\(\beta\)3 activity which downregulates the plasminogen activator inhibitor-1 (PAI-1) activity in fibroblasts\(^17\). The role of PAI-1 in hypertrophic scar formation is currently not understood, but it seems that its persistence may perpetuate the fibrotic process.

**Exaggerated Inflammation**

When injury has activated the clotting cascade, the kinin cascade, and the complement cascade, it will lead to the release of various vasoactive mediators and cytokines. They further stimulate the migration of inflammatory cells, such as neutrophils, macrophages, and T-lymphocytes, as well as epithelial cells, endothelial cells, and fibroblasts to the wound site\(^10\). Mast cells and the complement cascade are responsible for mediators that stimulate vasodilation. Kinins, complement factors, and trombin increase capillary permeability, which facilitates the extravasation of proteins into the wound site\(^8\). Subsequently, neutrophils and macrophages debride the wound and release several proinflammatory cytokines, some of which also are responsible for matrix production (Table 1)\(^1\). Burns or infected wounds exaggerate the inflammatory phase, which increases the concentration of potential profibrotic cytokines like TGF-\(\beta\), platelet-derived growth factor (PDFG) and interleukin-4. This may induce hypertrophic scar formation\(^6\).

**Platelets**

After aggregation, platelets degranulate to release an array of potent growth factors that principally function as chemotactic agents for the recruitment of
inflammatory cells (Table 1). Platelet aggregation and degranulation in fact mark the onset of inflammation. They release PDGF, TGF-β, basic fibroblast growth factor (FGF2), vascular endothelial growth factor (VEGF), hepatocyte growth factor (HGF), insulin-like growth factor (IGF) and epidermal growth factor (EGF). In addition to its chemotactic features, PDGF stimulates healing in non-healing wounds by enhancing the proliferation of fibroblasts and the production of ECM by these cells\textsuperscript{10,18}. Increased expression of PDGF may be involved in hypertrophic scar formation, in view of the presence of enhanced levels of this growth factor in hypertrophic scar tissue\textsuperscript{19,20}. However, PDGF seems to exhibit a dual effect in scar formation as it accelerates wound healing in the early stages and may induce fibrosis in the later stages\textsuperscript{20}.

Table 1: Activities and major cytokines of cells in this review

<table>
<thead>
<tr>
<th>Cells</th>
<th>Activities</th>
<th>Major cytokines</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelets</td>
<td>Inflammation</td>
<td>IL-1, TNF-α, PDGF, TGF-β, IGF-1, FGF2</td>
</tr>
<tr>
<td>Mast cells</td>
<td>Inflammation, ECM deposition</td>
<td>Histamine, TGF-β, TNF-α, IL-4, IL-13</td>
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<tr>
<td>Neutrophils</td>
<td>Inflammation, IL-1α, IL-1β, IL-6, TNF-α</td>
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</tr>
<tr>
<td>Macrophages</td>
<td>Inflammation, TGF-β, FGF2, IGF-1, IL-1, FGF10</td>
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<tr>
<td>T-lymphocytes (Th2)</td>
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</tr>
<tr>
<td>Langerhans cells</td>
<td>Reepithelialization, ECM deposition</td>
<td>FGF7, FGF10, IL-1α</td>
</tr>
<tr>
<td>Fibrocytes</td>
<td>Inflammation, ECM deposition</td>
<td>CCL2, CCL3, CCL4, CXCL1, CXCL8, IL-6, IL-10, TGF-β, PDGF, TNF-α</td>
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<td>Keratinocytes</td>
<td>Reepithelialization, ECM deposition</td>
<td>TGF-α, IL-1α, IL-6, VEGF</td>
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<td>Endothelial cells</td>
<td>Inflammation, Neovascularization, CCL2, FGF2, VEGF, CTGF</td>
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<tr>
<td>Fibroblasts</td>
<td>Reepithelialization, ECM deposition</td>
<td>FGF7, FGF10, IL-6, IGF-1, CTGF, TGF-β, IGF-1</td>
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Mast Cells

Mast cells are likely candidates to play a role in the etiology of hypertrophic scar formation\textsuperscript{21-23}. They are interspersed among dermal collagen bundles, and some studies showed larger numbers of mast cells in hypertrophic scars compared with normal scars\textsuperscript{24,25}, which is consistent with observations in other fibrotic diseases like...
fibrotic lung disorders and sclerodermatous skin lesions. However, in other studies no differences in mast cell numbers were found between both types of scars. It should be kept in mind that not only the number determines the role of mast cells, but also their activation state.

Mast cells can be stimulated by direct injury, IgE and various other proteins to expel their cytoplasmic granules containing histamine, proteoglycans, proteases, and cytokines that are involved in dermal matrix production (Table 1). Additionally, it is suggested that mast cells activate fibroblasts through gap junction intercellular communication. Histamine is capable of enhancing the formation of collagen by fibroblasts in vivo, and is significantly elevated in the plasma of patients developing hypertrophic scars compared with age-matched normal volunteers. Recently, hypertrophic scar mast cells were found to release more histamine than normal skin mast cells after stimulation by substance P, a neuropeptide. Mast cells are able to promote proliferation of fibroblasts by the release of TGF-β1, TNF-α and IL-4. This indicates that mast cells may play a role in hypertrophic scar formation via different mediators.

**Neutrophils**

Being highly motile, within a few minutes neutrophils congregate at the wound site. They are attracted by C5a and cytokines, including PDGF and CXCL8, expressed by activated endothelium and mast cells. Besides their capability of ingesting and destroying microorganisms and particles, neutrophils also produce proinflammatory cytokines, such as IL-1α and TNF-α that may be one of the first signals to activate local fibroblasts and keratinocytes by inducing the expression of FGF7. Although FGF7 stimulates epithelial cell proliferation, its role in scarring has not yet been determined. Unless a wound is highly infected, neutrophil infiltration ceases after a few days, and expended neutrophils are phagocytosed by tissue macrophages. Neutrophils can sustain the chemokine response and can be responsible for scarring, but their role in the development of hypertrophic scars is not yet clear.

**Macrophages**

Circulating monocytes are attracted to the wound site by cytokines like TGF-β, PDGF and monocyte chemotactic protein-1 (CCL2). At the wound site they transform into macrophages, peaking around 42 h after wounding. They not only perpetuate the inflammatory process, but also stimulate collagen production, angiogenesis and reepithelialization (Table 1). As such, macrophages play a pivotal role in the transition between the inflammatory and proliferative phase in which they coordinate and sustain the wound healing events.
Macrophages produce proinflammatory cytokines, including IL-1α, IL-1β, IL-6 and TNF-α, which are not only responsible for the control of inflammatory cell adhesion and migration, but also stimulate the proliferation of keratinocytes and fibroblasts. Macrophages can possibly initiate hypertrophic scar formation by production of TGF-β, PDGF, FGF2 and IGF-1, stimulating fibroblasts to produce excess collagen. IL-1 stimulates the release of matrix metalloproteinases (MMPs) and synergistically induces collagenase activity in conjunction with interferon (IFN)-γ and TNF-α released from inflammatory cells. Therefore, a decrease in wound levels of IL-1 may result in ECM accumulation and scar formation at the site of the injury. Such decreased levels of IL-1 are found in patients with hypertrophic scars. However, other studies showed an induced collagen production by IL-1, suggesting that IL-1 associated collagen production is a dose or time dependent process.

One of the most intensively investigated molecules associated with many types of fibrosis is TGF-β. TGF-β has three isotypes (TGF-β1, -β2 and -β3), all of which stimulate infiltration of inflammatory cells and fibroblasts and induce fibroblast proliferation, angiogenesis and synthesis of ECM. Additionally, TGF-β inhibits reepithelialization. TGF-β signals through transmembrane receptors that trigger intracellular regulatory proteins known as Smads. These transcription factors regulate transcription of target genes, including procollagen I and III. Particularly Smad3 is known as a mediator of fibrosis, which corroborates the observation that dermal fibrosis following irradiation is reduced in Smad3-deficient mice. Smad7 was recently recognized as an inhibitor of TGF-β/Smad3 signaling, thus either genetic or pharmacologic overexpression of Smad7 might be a potential target for hypertrophic scar therapy.

Many cells produce and respond to TGF-β during wound healing, but hypertrophic scar formation is principally associated with overexpression of TGF-β1 and TGF-β2 by macrophages, whereas TGF-β3 was shown to have antifibrotic properties. There is a strong and persistent expression of TGF-β mRNA, protein and their receptors in human hypertrophic scar fibroblasts and hypertrophic scar tissue after burns, while expression of decorin which inhibits TGF-β is downregulated. Moreover, serum of burn patients developing hypertrophic scars also contains higher levels of TGF-β compared with control patients. Accordingly, non-scarring foetal wounds show a reduced expression of TGF-β and a higher expression of fibromodulin, a TGF-β binding protein, compared with adult wounds. Treatment of foetal wounds with TGF-β1 and TGF-β2 caused a marked scarring of these wounds. Counteracting the effect of TGF-β1 and TGF-β2 by various methods significantly reduced scarring in several animal wound models. Moreover, adenoviral mediated overexpression of fibromodulin reduced wound scarring in vivo.
TGF-β is thought to promote fibrosis by mediating the transition of resident mesenchymal cells (including epithelial cells) into collagen-producing myofibroblasts and subsequent activation of (myo)fibroblasts. Additionally, TGF-β also reduces the collagenase mediated degradation of the wound matrix. In vitro, hypertrophic scar fibroblasts appear more responsive and sensitive to TGF-β stimulation compared with normal fibroblasts. In conclusion, the induced response of fibroblasts to TGF-β and the elevated levels of this cytokine make TGF-β and its downstream effector cells and molecules at least partially responsible for hypertrophic scar formation. Counteracting the effect of TGF-β1 and TGF-β2 and increasing expression of TGF-β3 may significantly prevent hypertrophic scar formation. However, it seems that it must be administered very early after (burn) wounding and a product is still not available.

Basic fibroblast growth factor (FGF2) stimulates migration, proliferation, and survival of various cells, including fibroblasts, endothelial cells, and keratinocytes, thereby promoting wound healing in general. FGF2 is expressed at the edge of human burn wounds, peaking between 4 and 11 days. Recently, it was found that postoperative intrallesional application of FGF2 might be successful in reducing scar formation in acute incisional wounds, possibly due to increased fibroblast apoptosis and decreased wound contraction. Silicone gel application increases FGF2 expression in hypertrophic scars as well, which may be associated with its scar reducing effect. However, although FGF2 application on burn wounds did improve scar quality, it did not prevent hypertrophic scar formation.

T-lymphocytes

T-lymphocytes play a noteworthy role in wound healing. They are mobilized to the wound site, are activated by antigens, and subsequently produce cytokines that further activate macrophages and other inflammatory cells. Probably attracted by IL-15, CXCL8, and CCL2, T-lymphocytes are found in high numbers at wound sites that result in hypertrophic scars. The infiltration of T-lymphocytes in the early wound, particularly CD4+ T helper-2 (Th2) cells, has been strongly linked to fibrogenesis. By contrast, a T helper-1 (Th1) response leads to reduced fibrogenesis. Th2 cells produce various cytokines, including IL-4, IL-5, IL-6, IL-10, IL-13, and IL-21, by which they activate and direct other immune cells to engage in the wound healing process. In burn patients, hypertrophic scar formation was found to be associated with a polarized Th2 response together with increased serum levels of IL-4, IL-6, and IL-10, and decreased levels of IFN-γ and IL-12, compared with healthy control patients. IL-4 seems a strong profibrotic mediator, which is in line with increased levels of IL-4 found in hypertrophic scars and other fibrotic tissues. IL-4 acts on Th2 cells to promote further production of cytokines (auto-regulation), but also stimulates
fibroblasts to produce collagen and fibronectin. Neutralizing antibodies to IL-4 reduced dermal collagen deposition in various mouse models. IL-13 affects immune cells in the same way as IL-4, since they mainly share the same signaling pathways. In the development of various types of fibrosis IL-13 is found to have a dominant role by stimulating the production of TGF-β1 in macrophages. However, the role of IL-13 in the pathogenesis of hypertrophic scars is not well understood at this moment.

Incompatible with the theory that a Th2 response is simply profibrotic, is the fact that IL-10, which is part of the Th2 response, is an anti-inflammatory cytokine. It is capable of inhibiting the synthesis of many pro-inflammatory cytokines including IFN-γ, IL-1, and TNF-α, and both CC and CXC chemokines by macrophages, thereby limiting ongoing immune responses and inflammation. Accordingly, foetal wound repair, which is characteristically scarless, has been found to result in scar formation in IL-10 deficient mice, which suggests that IL-10 is a necessary component of scarless foetal wound repair. The Th1 response includes production of IFN-γ, which increases levels of IL-12 by macrophages, but also promotes and preserves the Th1 response by increasing the production of IFN-γ via auto-regulation and inhibiting the production of Th2-derived IL-4. Although some attempts have been made to decrease collagen deposition by promoting a Th1 response, its effect is disputable.

**Fibrocytes**

Scientists investigating fibrogenesis and hypertrophic scar formation recently gained interest in blood-born fibroblast-like cells called fibrocytes. Circulating fibrocytes migrate to wound sites during the inflammatory phase, and are capable of producing proinflammatory cytokines, chemokines, growth factors (Table 1) and ECM, including fibronectin, collagen type I, and type III. Ultimately, fibrocytes may be a precursor of myofibroblasts. Several studies suggest that fibrocytes are implicated in wound healing, fibrotic tissue repair, and hypertrophic scar formation.

In hypertrophic scar tissue, fibrocytes are present in higher numbers compared with mature scar tissue. Given that TGF-β1 increases the differentiation and functional activity of fibrocytes, and that serum of recovering burn patients contains higher levels of TGF-β compared with healthy control patients, it is suggested that the fibrocyte differentiation and activity is upregulated in (burn) patients that develop hypertrophic scars. They may contribute to the excessive ECM deposition responsible for hypertrophic scar formation.
Chapter 2

Prolonged Reepithelialization

Reepithelialization is initiated as soon as possible to provide a protecting epidermal layer to prevent infection and excessive water loss. Adequate reepithelialization is important since scar hypertrophy is more likely to occur if wound closure requires more than three weeks. In full thickness (burn) wounds, keratinocytes from the wound edges invade the denuded area, whereas in partial thickness wounds, cells from the epithelial appendages help to speed up wound closure. Keratinocytes migrate, proliferate, and differentiate in an intensive and intimate crosstalk with dermal cells, mediated by soluble autocrine and paracrine acting factors.

After wounding, stored IL-1α is released by keratinocytes, which activates fibroblasts and adjacent keratinocytes, and attracts endothelial cells and lymphocytes to the injured area. Fibroblasts in turn secrete FGF7 and granulocyte-macrophage colony-stimulating factor (GM-CSF), to further activate basal and suprabasal keratinocytes. The activation is sustained by other growth factors like TNF-α, members of the EGF family (TGF-α, EGF, HG-EGF), CXCL8 and IFN-γ from mast cells, monocytes, macrophages and keratinocytes in the wound bed. The activated state normally ceases when the wound is reepithelialized, but in hypertrophic scars keratinocytes remain activated.

Following activation, keratinocytes migrate in response to EGF, HB-EGF, FGF7, TGF-α, TGF-β and plasmin stimulation, guided towards fibrin, fibronectin, vitronectin and collagen bundles present beneath the blood clot. They cut their way through the blood clot barrier by production of tPA and urokinase-type plasminogen activator (uPA) to induce transformation of plasminogen in plasmin, that in turn degrades the fibrin network. To further crawl between the collagen bundles, keratinocytes produce MMP-1 and MMP-9.

Around a day after injury, keratinocytes start to proliferate under influence of cytokines like TGF-β1, HB-EGF, EGF, TGF-α, HGF, NGF, FGF7, VEGF, CXCL1, IL-6, and GM-CSF from macrophages, fibroblasts, and keratinocytes. Without signals from fibroblasts, keratinocytes are not able to re-establish a functional epidermis. Following proliferation, keratinocytes differentiate through FGF7 and GM-CSF stimulation. In contrast with the activation in the early wound healing phases, TGF-β from the fibroblasts finally induces keratinocytes to return to an inactivated state, whereas keratinocytes in turn decrease collagen production by fibroblasts.

In hypertrophic scars, the keratinocytes show increased proliferation and differentiation compared with normal scar keratinocytes. This activated epidermis has reduced IL-1α and increased PDGF expression compared with normal...
scar tissue, which probably directly affects collagen production by fibroblasts. In cell co-culture studies, activated keratinocytes from keloids induced connective tissue growth factor (CTGF), TGF-β, and VEGF expression in normal skin fibroblasts and increased proliferation and collagen production. In another cell co-culture study, hypertrophic scar derived keratinocytes also induced collagen production in fibroblasts. Unfortunately, in these studies normal skin keratinocytes were used as controls where normal scar tissue cells would be desirable.

How the keratinocytes remain activated is not known, but increased presence of epidermal Langerhans cells in hypertrophic scars indicates that immunologic processes are involved. The epidermal-dermal crosstalk in relation with excessive scar formation becomes even more complicated by the fact that epidermal cells can transform in collagen producing mesenchymal cells. Especially in tumorgenesis these processes have recently been described as a source of excessive matrix deposition.

**Overabundant Extracellular Matrix Deposition**

After approximately 4 days of wound debridement by inflammatory cells, the violated dermis starts to repair by the formation of granulation tissue. Stimulated by macrophages, fibroblasts and endothelial cells start to migrate over the provisional wound matrix into the wound space, assisted by MMPs. Eventually, the fibrin clot is transformed into connective tissue rich in blood vessels, underlying the granular appearance. This transformation requires a balance between matrix degradation and production, since deficient degradation or excessive production of ECM leads to hypertrophic scar formation.

Fibroblasts are primarily responsible for the formation of granulation tissue, which consists of collagens, glycosaminoglycans, and proteoglycans. Migration of fibroblasts to the wound area is stimulated by PDGF, NGF, TGF-β, and CTGF. Fibronectin facilitates the migration of fibroblasts and functions as a binding site for ECM components. Mechanical forces and TGF-β1 stimulate fibroblast differentiation into myofibroblast. This fibroblast subtype is characterized by α-smooth muscle actin (α-SMA) expression and is involved in wound contraction. Compared with normal dermal fibroblasts, myofibroblasts produce higher amounts of ECM components. In hypertrophic scar tissue, the density and proliferation activity of myofibroblasts is increased, which presumably leads to an overproduction of ECM. It is now thought that these myofibroblasts have different origins. In addition to resident mesenchymal cells, myofibroblasts can originate from epithelial cells via epithelial-mesenchymal-transition. Blood-born fibroblast-like cells, called fibrocytes, also may be myofibroblast precursor cells, invading the wound site during the inflammatory phase.
Figure 1: Numerous cells are directly and indirectly involved in the activation of (myo)fibroblasts by higher (↑) or lower (↓) expression of various factors compared to normal scar formation. The (myo)fibroblasts in turn produce excess extracellular matrix.

Hyaluronan (HA) is a prominent component of the ECM during the early phase of wound healing, contributing significantly to cell proliferation and migration132. HA production by (myo)fibroblasts is stimulated by PDGF133. In adult wound healing, HA levels return to baseline after about 2 weeks, whereas levels remain high in foetal wounds134. It seems that HA limits the deposition of the ECM, and collagen in particular, thereby contributing to scarless healing in the foetus134,135. Accordingly, HA grafts appear to produce a foetal-like environment with reduced TGF-β1 expression and consequently may reduce scar formation in adults136,137.

Collagen production by (myo)fibroblasts is stimulated by various growth factors, including PDGF, TGF-β1, and TGF-β2, EGF, IGF-1, FGF2, CTGF and cysteine-rich 61 (Cyr61)10. The roles of PDGF, TGF-β, and FGF2 have been discussed above. Another
suggested growth factor in the pathogenesis of hypertrophic scars and other fibrotic diseases is CTGF \(^{138}\). Recently, hypertrophic scar fibroblasts were found to have increased intrinsic CTGF expression compared with normal fibroblasts. Furthermore, hypertrophic scar fibroblasts produced more CTGF after TGF-\(\beta\) stimulation \(^{139}\). CTGF is produced by fibroblasts and endothelial cells. It stimulates their own chemotaxis and proliferation \(^{10}\), and is thus implicated in ECM production and angiogenesis \(^{140}\). As a downstream mediator of some of the effects of TGF-\(\beta\)1 on fibroblasts, CTGF stimulates the production of ECM, including fibronectin and type I collagen \(^{141,142}\). Finally, CTGF is capable of regulating both MMPs and their inhibitors (TIMPs), thus controlling the integrity and stability of the ECM \(^{140}\). Therefore it seems that CTGF is important for granulation tissue and subsequent scar formation.

Angiotensin (ANG) II is the biologically active component of the renin-angiotensin system (RAS). It is not only involved in the regulation of the blood pressure, but also in wound healing. It stimulates the production of the ECM, induces (myo)fibroblast proliferation \(^{143}\) and differentiation \(^{72}\), and fibroblast and keratinocyte migration \(^{144}\). Several studies have shown the involvement of the RAS in fibrotic processes in many organs (heart, liver, kidney), and in cutaneous scar formation as well \(^{145}\).

ANG II has proinflammatory activity as it induces TGF-\(\beta\) expression and directs ECM synthesis. It is formed through cleavage of ANG I by angiotensin-converting enzyme (ACE). ACE inhibition and receptor blockade are clinically used for many disorders, such as renal disease, hypertension, and congestive heart failure. Interestingly, ACE inhibition reduces TGF-\(\beta\) expression \(^{146}\). Furthermore, application of the ANG II analog NorLeu3-A(1–7) has positive effects on cutaneous wound healing and reduces scar formation \(^{147,148}\). ANG II exerts its effects by binding to one of the two main receptors (AT1 and AT2). Binding to either one of these receptors often has opposing effects. Therefore, the definitive outcome of ANG II action depends on the presence of one, or the balance between the two receptors. It is thought that the AT1 receptor stimulates collagen production and that the AT2 receptor has antifibrotic effects \(^{149}\). During wound healing in the rat, AT1 is downregulated while AT2 is upregulated \(^{150}\). Specific inhibition of one of these receptors might be effective to reduce scarring without influencing the positive effects of the RAS on wound healing.

**Augmented Neovascularization**

New blood vessel formation is an essential process in wound healing that mainly presents as angiogenic sprouting of preexisting capillaries during early granulation tissue formation. In burn wounds treated with a split-thickness skin graft, revascularization will initially take place by inosculcation of the capillary network of the graft, which seems essential for graft survival \(^{151}\). The grafted capillaries are
eventually replaced by new ones. The major cytokines that stimulate and regulate angiogenesis during wound healing appear to be VEGF, FGF, angiopoietin, and TGF-β. New vessels invade and re-populate the wound in unison with macrophages, fibroblasts, and keratinocytes as a source of new ECM constituents and a plethora of other (growth) factors152. In hypertrophic scar tissue, blood flow is increased as measured by laser Doppler153, which implies the presence of excess of microvessels compared with their density in normal scars.

To initiate angiogenic sprouting, destabilization of the existing capillaries by angiopoietin-2 is a necessary first step. In response to inflammatory stress, endothelial cells release angiopoietin-2 that competes with constitutive angiopoietin-1 for the receptor Tie-2, a transmembrane tyrosine kinase154. Next, for angiogenesis to occur, the endothelial cells in existing capillaries need to create intercellular space with concurrent increase in vascular permeability. Subsequently, MMPs degrade the pre-existing basal lamina, which is followed by endothelial proliferation and migration from the original capillary into the tissue. Connection to other (preexisting) vessels before becoming functionally perfused again is accompanied by vascular maturation in which pericytes as vascular support cells play an important role. These pericytes may also produce collagen155,156 and are able to differentiate to myofibroblasts as well157. During the later stages of wound healing, vascular regression allows return to a level of perfusion that is sufficient for tissue homeostasis.

Various studies addressed the role of angiogenesis on the wound healing process per se in animal models, yet information on its relation to hypertrophic scar formation are scarce. Besides reduced oxygen concentration in hypertrophic scar tissue158, inflammatory processes contribute to a condition that favours angiogenic sprouting in the late granulation stage. The vessel density in hypertrophic scar tissue is higher than in normal skin and vessels showed a more dilated phenotype159, which may find its origin in ongoing neovascularization, insufficient vasoregression, or a combination of the two. Nonetheless, the observation that at 52 weeks after breast surgery, microvascular density remained elevated160 indicates that even when wound healing is non-pathologic, a normal situation is only achieved after a long-term active remodeling process.

VEGF is considered to be instrumental for endothelial cells to further engage in new sprout formation, as it directly affects vascular permeability and temporary fibrin matrix formation, endothelial cell migration, proliferation, and survival161. Serum levels of VEGF quickly rise in patients with burn wounds162 and local rise in production is also implicated in the formation of granulation tissue163. Chronic non-healing wounds are associated with increased plasmin inactivated VEGF levels as
well as with increased soluble VEGFR-1 that neutralizes its angiogenic activity\textsuperscript{164}. Its involvement in wound healing is, however, quite complex. Uniform well healed wounds after partial thickness burns, with an architecturally normal epidermis and a lower uniform change in wound blood flow, are associated with lower levels of VEGF\textsuperscript{165}. In contrast, a hypercellular, overkeratinized and thickened epidermis is associated with higher wound blood flow throughout the wound healing period and higher levels of VEGF\textsuperscript{165}. Several animal studies suggested that both angiopoietin-1 and VEGF can serve as exogenous stimulators of wound healing\textsuperscript{166,167}, but to what extent dysbalances in local expression of these factors accompanies hypertrophic scar formation is at present unknown.

**Atypical Extracellular Matrix Remodeling**

Derailment of the wound healing process becomes tangible in the remodeling phase when the ECM matures and abnormal architecture becomes apparent. The mechanical function of ECM greatly depends on the architecture of the collagen network formed, the assembly of collagen fibrils and the maturation into larger structures. In the mean time, the nature and structure of the proteoglycans change\textsuperscript{168}.

During the remodeling phase, (myo)fibroblasts normally replace HA by proteoglycans such as decorin, which binds TGF-β1 and regulates collagen fibrillogenesis\textsuperscript{169}. In hypertrophic scars, fibroblasts synthesize less decorin than normal dermal fibroblasts\textsuperscript{170} and expression of decorin in burn scars is suppressed for about 12 months\textsuperscript{55}. This may influence hypertrophic scar formation, since decorin was recently found to inhibit cell proliferation and downregulate TGF-β1 production and type I collagen synthesis in hypertrophic scar fibroblasts\textsuperscript{171}.

In the skin, collagen fibrils are composed of both type I and III collagen, in which type III comprises almost 20% of the total amount of collagen\textsuperscript{172}. It is thought that type III collagen plays a role in the fibrillogenesis and determines the collagen fibril diameter\textsuperscript{173}. During granulation tissue formation, type III collagen expression increases more than the type I expression, resulting in an altered ratio between the two collagen subtypes from 20% to 50% type III collagen\textsuperscript{174}. During maturation of the scar the ratio decreases again to normal levels. However, expression ratios of both collagen subtypes remain high in hypertrophic scars\textsuperscript{54}.

The role of type III collagen in wound healing was shown in experiments with type III collagen deficient mice. These mice displayed very severe spontaneous skin wounds and the diameters of the collagen fibrils of their skin were not uniform, as thinner and thicker fibrils were observed compared with normal skin\textsuperscript{173}. Self-assembly in vitro experiments with different ratios of type III to type I collagen showed that an increase in type III collagen concentration results in a decreased fibril diameter\textsuperscript{175,176}. 
Collagen structure is partly determined by the type of crosslinks between the collagen molecules in the fibril. Depending on the function and mechanical load of the tissue, different types of crosslinks are found\textsuperscript{177}. In bone and cartilage a specific pyridinoline crosslink is present, in which hydroxylated lysyl residues of the telopeptides of the collagen molecules are involved. This type of crosslink is absent in normal skin, but present in various pathological conditions of skin fibrosis, including hypertrophic scars\textsuperscript{178}. The telopeptide lysyl hydroxylase, LH2b, plays a determinative role in the formation of these bone-like crosslinks\textsuperscript{179-181} and influences fibril formation, as overexpression of this enzyme results in thinner fibrils\textsuperscript{182}. Collagen fibres crosslinked via the LH2b dependent pathway are less susceptible to degradation by MMP-1\textsuperscript{183}. TGF-β treated fibroblasts, myofibroblast, and (myo)fibroblasts derived from hypertrophic scars show increased LH2b expression levels\textsuperscript{184,185}. Inhibition of this crosslinking pathway might improve wound healing by making the newly formed collagen more susceptible for degradation.

In addition to the above-described collagen accumulating characteristics of myofibroblasts, it is known that hypertrophic scar myofibroblasts have reduced collagen degrading characteristics. Turnover of ECM is controlled by the balance between MMPs and their inhibitors, TIMPs. Hypertrophic scar myofibroblasts show decreased expression levels of MMPs and increased expression levels of TIMPs\textsuperscript{186-188}, thereby favoring ECM accumulation the combination of easier degradable collagen and increased expression of proteolytic enzymes may be an approach for treatment of hypertrophic scars after they have already been formed.

**Reduced Apoptosis**

In conjunction with ECM deposition and remodeling, the skin reduces its defect by contraction. Myofibroblasts, which express α-SMA in response to PDGF and TGF-β stimulation, are primarily responsible for this process\textsuperscript{129}. When contraction is completed as the wound is fully epithelialized, the myofibroblast phenotype normally disappears. In hypertrophic scars, however, they tend to persist, which may represent an important element in the mechanisms of excessive ECM deposition and scar contractures as seen during hypertrophic scar formation.

Programmed cell death is suggested to be primarily responsible for the decrease in cell number throughout the various phases of wound healing\textsuperscript{189}. In this way superfluous inflammatory cells are eliminated after wound debridement, giving space for granulation tissue formation. When sufficient ECM is formed, apoptosis of myofibroblasts is desirable, and normally peaks around 25 days after wounding. In view of the hypercellularity of hypertrophic scar tissue\textsuperscript{130,131}, decreased gap junctional intercellular communication of hypertrophic scar fibroblasts\textsuperscript{190}, and increased levels
of the bcl-2-proto-oncogene in the peripheral blood mononuclear cell fraction of burn patients\textsuperscript{181}, it is plausible that decreased apoptosis of myofibroblasts leads to an imbalance between ECM deposition and degradation, resulting in hypertrophic scars\textsuperscript{189,192}. Ultimately, it may underlie the dreaded contractures that are seen in many burn patients. However, conflicting results are presented in the literature. Some authors indeed find decreased apoptosis together with higher resistance to apoptotic inducers in hypertrophic scar myofibroblasts\textsuperscript{193,194}, while others find an increased rate of apoptosis\textsuperscript{130,195,196}.

Many genes are associated with apoptosis. A key member of the apoptotic pathway is p53, a transcription factor that regulates the cell cycle\textsuperscript{196}. Disruption to the regulation of p53 will result in impaired apoptosis. Surprisingly, expression of p53 is found to be higher in hypertrophic scars compared to normal scars, and seems to be related with scar maturation\textsuperscript{196}. Furthermore, significantly higher levels of activated caspase-3, which is only activated during apoptosis, are found in hypertrophic scar tissue compared with normal scar tissue\textsuperscript{195}. While a gene mutation of p53 is reported to be present in keloids\textsuperscript{197}, the meaning of the elevated p53 expression in hypertrophic scars cannot be explained at this moment. In conclusion, the apoptosis rate of hypertrophic scar fibroblast is probably associated with the tissue environment and ultimately, the balance between cell proliferation and apoptosis seems most important.

**Conclusion**

It seems that a wide array of wound healing processes is involved in hypertrophic scar formation (Table 2). Platelets, macrophages, T-lymphocytes, mast cells, Langerhans cells, and keratinocytes are directly and indirectly involved in the activation of (myo)fibroblasts, which in turn produce excess ECM (Fig. 1). Once the hypertrophic scar trail is taken, the involved cells jointly create and maintain an environment that promotes the scar phenotype.

All cells strongly respond to local changes and quickly change through environmentally imprinted behavior. Intricate interactions in scar tissue, both via cell-cell contact and via secreted products, are spatiotemporally controlled and difficult to mimic in in vitro cell systems, thereby largely hampering extrapolation of in vitro observations to the wound in a human patient. Yet the knowledge created in simplified in vitro, ex vivo, and animal models in conjunction with observations in patients helps to more closely define which molecular and cellular features are implicated in hypertrophic scar formation in humans. However, studying cell (dys)function in the complex microenvironment of the wound remains a prerequisite.
Table 2: Major derailed processes potentially causing hypertrophic scar formation

<table>
<thead>
<tr>
<th>Cells</th>
<th>Process</th>
<th>Effects</th>
</tr>
</thead>
<tbody>
<tr>
<td>Platelets, macrophages, (myo)fibroblasts</td>
<td>Increased fibronectin expression&lt;sup&gt;13&lt;/sup&gt;</td>
<td>Increased fibroblast migration</td>
</tr>
<tr>
<td>Mast cells</td>
<td>Increased histamine release&lt;sup&gt;30,35,36&lt;/sup&gt;</td>
<td>Increased fibroblast collagen formation</td>
</tr>
<tr>
<td>Platelets, macrophages</td>
<td>Increased TGF-β and PDGF expression&lt;sup&gt;20,40,50,51&lt;/sup&gt;</td>
<td>Increased fibroblast proliferation, differentiation and ECM deposition</td>
</tr>
<tr>
<td>T-lymphocytes</td>
<td>Polarized Th2 response with increased IL-4 and IL-13 expression&lt;sup&gt;74-77&lt;/sup&gt;</td>
<td>Increased ECM deposition</td>
</tr>
<tr>
<td>Fibrocytes</td>
<td>Increased migration&lt;sup&gt;93,96,97&lt;/sup&gt;</td>
<td>Increased myofibroblast density</td>
</tr>
<tr>
<td>Keratinocytes</td>
<td>Prolonged activation&lt;sup&gt;20,107,108,118,124&lt;/sup&gt;</td>
<td>Increased fibroblast proliferation and ECM deposition</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>Increased ACE expression&lt;sup&gt;145&lt;/sup&gt;</td>
<td>Increased fibroblast proliferation, differentiation and ECM deposition</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>Increased CTGF expression&lt;sup&gt;138,139,141&lt;/sup&gt;</td>
<td>Increased ECM deposition and angiogenesis (downstream TGF-β)</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>Increased LH2b expression&lt;sup&gt;178,184,198&lt;/sup&gt;</td>
<td>Formation of pyridinoline crosslinks</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>Decreased MMP and induced TIMP expression&lt;sup&gt;180-188&lt;/sup&gt;</td>
<td>Reduced collagen breakdown</td>
</tr>
<tr>
<td>Fibroblasts</td>
<td>Decreased apoptosis&lt;sup&gt;189-194&lt;/sup&gt;</td>
<td>Increased ECM deposition and scar contraction</td>
</tr>
</tbody>
</table>

It remains unclear whether the discussed molecular and cellular changes are a cause or a consequence of unusual scar tissue formation. People may even have predisposed differences in cytokine expression, to influence hypertrophic scar formation. However, derailed immunological responses early following wounding seem to be important. In burn patients, a systemically upregulated immune response may influence the quantity and phenotype of inflammatory cells and mediators that migrate to the wound area, causing an exaggerated inflammatory phase of wound healing<sup>75</sup>. This possibly initiates a cascade that disturbs the subsequent wound healing processes and eventually leads to overabundant ECM production. To develop preventive treatment modalities, one should aim to put the early wound healing back on track as quickly as possible, in order to influence the subsequent continuous and overlapping processes. However, forcing altered, perhaps predisposed, conditions to change to “normal” levels may influence other aspects of wound healing.

Indeed, further research on the cellular and molecular mechanisms of wound healing and hypertrophic scar formation is particularly important, in order to be able to influence the evolution of hypertrophic scars towards normal scars in the future.
References

Potential cellular and molecular causes of hypertrophic scar formation


Potential cellular and molecular causes of hypertrophic scar formation


