Review

Possible preventive treatments of hypertrophic scar formation

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ABSTRACT

Scar formation is a normal outcome of wound healing. However, after trauma, surgical injury and especially in burn wounds, hypertrophic scar formation can occur. This is a process of abnormal wound healing resulting in raised, red scars. Patients with hypertrophic scar formation are presented with itching, pressure, pain and cosmetical concerns. It is believed that hypertrophic scar formation is caused by an exaggerated inflammatory response, stimulating fibroblasts to produce excessive ECM. Abnormal ECM composition and increased ECM production result in hypertrophic scars. Current treatments of HSF are inefficient and variable. However still incomplete, our understanding of the etiology of hypertrophic scar formation has lead to new targets for preventive treatments. In this review, mechanisms in normal wound healing and the altered mechanisms in hypertrophic scar formation are described. Potential targets are summarized and the possibilities of preventive therapies using these targets are discussed.

1. Introduction

The skin is an important barrier between internal and external environment. Besides protection against pathogens, its functions contain thermoregulation, sensation, storage of nutrients and prevention of dehydration. When the barrier of the skin is broken, it is crucial that cutaneous integrity is re-established. This process of wound healing involves complicated cellular interactions between inflammatory cells, endothelial cells, keratinocytes and (myo)fibroblasts.

Scar formation is a normal result of wound healing. In adults, true regeneration of the skin is never established; scar formation always occurs in some degree in the adult wound healing process. The difference between normal skin tissue and scar tissue is mainly the composition of extracellular matrix (ECM). Scar tissue is weaker than normal skin tissue.

In humans, two major forms of excessive scarring are seen; keloids and hypertrophic scar formation. The main difference between these forms is that keloids extend beyond the margins of the original wound, whereas hypertrophic scars do not. This review is focused on the pathogenesis and treatment of hypertrophic scar formation.

Hypertrophic scar formation is a process of abnormal wound healing which occurs after trauma or surgical injury, but most often occurs in burn victims. Hypertrophic scars are characterized by raised tissue, color mismatch, rough texture and stiffness. Next to cosmetical concerns, patients with hypertrophic scars are presented with itching, pressure and pain [1]. By their appearance, hypertrophic scars can easily be distinguished from normal scars. More than in 'normal scars', the ECM composition in hypertrophic scars differs from the ECM in healthy skin tissue. Also, there is an increased vascular density in hypertrophic scars. These characteristics of hypertrophic scars and their potential causes will be described in the second section of this review.

The last 60 years, treatment of patients with burn wounds which would previously have been fatal, has greatly improved. The development of treatments like fluid resuscitation, antibiotics and aggressive surgical debridement have all contributed to an increased survival of both burn and trauma victims. However, the development of treatments of hypertrophic scars after their formation is relatively slow [3].

Current treatments of hypertrophic scars after the healing process include corticosteroids, topical silicone gel and surgical procedures like Z- or W-plasty and grafting [2]. In the prevention of hypertrophic scar formation (HSF), dermal substitution is the most frequently used treatment. However, research has shown that the long-term results of dermal substitution are modest [4]. If the underlying cellular and molecular mechanisms of HSF are identified, preventive treatments can be developed.

Overall, the wound healing process can be divided in three stadia: the inflammatory phase, followed by the granulation/proliferative phase and finally the scar–remodeling phase/resolution phase. In all these three stadia, healing processes in HSF differ from processes in 'normal' scar formation. It is still unclear which processes are a cause and which are a consequence of the pathogenesis.

Rising evidence exists that in the early stages of HSF exaggerated inflammation leads to (over)stimulation of (myo)fibroblasts, which as a consequence produce overabundant extra-cellular matrix (ECM). This excessive ECM production by fibroblasts is believed to be the main cause of HSF. In the first part of this review I will summarize mechanisms of normal wound repair, and give an overview

of alterations in these mechanisms which could give rise to the pathogenesis of HSF. Then the most commonly used treatments used for HSF are summarized. Ultimately, possible targets for future preventive therapies are discussed.

2. Cellular and molecular mechanisms in normal wound healing and scar formation

The process of wound healing is complex and many molecular and cellular mechanisms are involved. In the literature the healing process is divided into three phases. It should be kept in mind that the definitions of the different phases are quite arbitrary, and are not reflecting the true continuum present in the normal wound healing process. The whole process of healing differs with age, severity of the wound, and the location of the wound.

The first step in wound healing is the formation of a blood clot shortly after injury. Inflammatory cells are subsequently attracted to the wound site, initiating the inflammatory phase. The most important function of the inflammatory cells in this phase is the protection against pathogens. Additionally, the inflammatory cells produce cytokines and growth factors which lead to the chemotaxis of fibroblasts, keratinocytes, endothelial cells and other inflammatory cells. The functions of these cells are ECM production, reepithelialization and the formation of new blood vessels, respectively. Migration and proliferation of fibroblasts, keratinocytes and endothelial cells continues in the proliferative phase. Connective tissue containing ECM, fibroblasts and blood vessels is formed, beneath a newly formed epithelium. The wound healing process is completed during the resolution phase, also called scar-remodeling phase, in which the remodeling of the produced ECM and regression of vascular density takes place.

In Figure 1, a schematic representation of the wound healing process is shown. In this figure, three points in time represent different stages of normal wound healing. Figure 1A shows the blood clot formation and represents the beginning of the inflammatory phase. Figure 1B shows reepithelialization and formation of granulation tissue, representing the proliferation phase. Figure 1C can be seen as a transition between the proliferative phase and the resolution phase/ scar remodeling phase.

In the following section the mechanisms and processes in the inflammatory, proliferative and the scar-remodeling phase are described. Of each phase, the involved cells and cytokines are summarized. The density and activity of some cells is found to be increased in HSF. Also increased amounts of certain cytokines and growth factors are associated with HSF. The role of these cells and cytokines is discussed in the next section.

2.1 Inflammatory phase

2.1.1. Blood clot formation

Shortly after blood vessels are injured in the wound area, the vessels constrict and platelets aggregate at the site of injury. This aggregation leads to initiation of the coagulation cascade and the complement cascade. Ultimately, these cascades lead to the formation of a blood clot consisting of cross-linked fibrin, fibronectin, vitronectin and trombospondin.

The blood clot plays an important role in the attraction of cells responsible for wound repair. Fibronectin and fibrin stimulate migration of fibroblasts and endothelial cells to the wound area [7]. In addition, the complement cascade which is initiated during the blood clot formation leads to release of cytokines promoting the migration of inflammatory cells to the wound site [8].

After their aggregation, platelets in the blood clot can degranulate. The growth factors PDGF, TGF-β IGF-1 and FGF2 are released which contribute to the attraction of inflammatory cells, such as neutrophiles, macrophages and T-lymphocytes. The role of these inflammatory cells in wound healing will be discussed later.

In normal wound healing, the blood clot is degraded by endothelial cells while the inflammatory phase continues. Tissue-type plasminogen activator (tPA) is produced by endothelial cells, which are attracted to the blood clot by fibrin and fibronectin. The produced tissue-type plasminogen activator converts plasminogen to plasmin, which in turn degrades the fibrin network [9]. The side products of fibrin degradation also serve as chematactants of neutrophiles and monocytes [10].

2.1.2. Fibroblast migration and differentiation

Fibroblast migration and activation is very important in wound healing. The migration of dermal fibroblasts from the intact adjacent skin is stimulated by fibronectin and cytokines produced by inflammatory cells and other resident cells [7] [26]. Migration of fibroblasts is initiated by expression of *de novo* contractile bundles containing cytoplasmic actins. Once present at the wound site, fibroblasts produce collagen and other components of the ECM. Possible other origins of fibroblasts have been proposed. Fibrocytes, which are blood-borne leukocyte cells with fibroblast characteristics, have been found to be able to differentiate into fibroblasts [27] [28], as well as pericytes and smooth muscle cells from adjacent blood vessels.

Eventually the fibroblasts at the wound site can further differentiate into myofibroblasts, which have a more active ECM production [30] and also are responsible for wound contraction. Myofibroblasts express α-smooth muscle actin which enables stronger contractile forces than the

cytoplasmic actin containing bundles in migrating and activated fibroblasts [29]. The differentiation of fibroblasts into myofibroblasts is induced by mechanical properties of the micro-environment and by TGF-β. Myofibroblasts are responsible for wound contraction in the early phases of the wound healing process, which is essential for normal wound healing.

2.1.3. Inflammatory response

When the blood clot formation is complete, migration of inflammatory cells to the wound site is stimulated. This stimulation is mediated by the complement cascade, fibronectin, fibrin, the side-products of fibrin degradation and also by growth factors released by platelets in the blood clot. The migration of these inflammatory cells is further stimulated by increased permeability and vasodilatation of blood vessels near the wound. Vasodilatation is caused by mast cells and the complement cascade. Increased permeability is achieved by kinins, complement factors and thrombin [11]. Finally, mast cells, neutrophiles, macrophages and T-lymphocytes are mobilized to the wound site. The main function of these inflammatory cells in wound healing is the protection against pathogens. However, cytokines produced by neutrophiles and macrophages have been found to be responsible for ECM deposition [12].

2.1.4. Neutrophiles and macrophages in wound healing

Neutrophiles may be involved in the activation of fibroblasts and keratinocytes. This activation is mediated by the release of IL-1α and TNF-α, stimulated by FGF7 [13]. IL-1α and TNF-α are also produced by macrophages. Thereby macrophages contribute to the activation of keratinocytes and fibroblasts. Also, macrophages can stimulate fibroblasts to produce collagen via the release of the growth factors PDGF, FGF2, IGF-1 and TGF-β [14].

Neutrophiles are present in the early stages of wound healing, the first two days after the injury. After two days, neutrophil migration stops and remaining neutrophiles are phagocytosed by macrophages. Macrophages are abundant from day 3-7 after injury. In this period the fibroblast migration and activation is induced and the formation of granulation tissue is started. There is a transition between the inflammatory phase and the proliferative phase of wound healing, in which macrophages play an important role [12].

2.2 Proliferative phase

2.2.1 Keratinocyte and fibroblast migration and proliferation

The first step of the proliferative phase in wound healing is migration of keratinocytes from the wound edges and subsequently their proliferation. Also, in the proliferative phase fibroblasts are mobilized, activated and start to proliferate, stimulated by inflammatory cells as described above. Fibroblasts and the produced ECM together form the basis for newly formed connective tissue called granulation tissue. The name 'granulation tissue' originates from the granular appearance of the tissue. This granular appearance is caused by the presence of many newly formed blood vessels.

The proliferation of keratinocytes leads to reepithelialization of the wound, which is important to prevent infection and water loss. There is an intimate cross talk between fibroblasts and keratinocytes during wound healing, which is tuned by a balance between a pro-inflammatory cytokine mediated environment and a TGF- β mediated environment. This interaction between fibroblasts and keratinocytes is in a double paracrine manner [15] and without this interaction reepithelialization is not possible [17].

Keratinocytes at the wound edges release stored IL-1 after injury. This leads to activation of adjacent keratinocytes and also fibroblasts, which in turn produce FGF7 and granulocyte-macrophage colony stimulating factor (GM-CSF). FGF7 and GM-CSF stimulate basal and supra-basal keratinocytes [16]. Their proliferation is also stimulated by cytokines produced by other keratinocytes and macrophages, such as TGF-β1, HB-EGF, EGF, TGF-α, HGF, NGF, VEGF, CXCL1 and IL-6.

The migration of keratinocytes is guided towards fibrin, fibronectin, vitronectin and collagen bundles beneath the blood clot by EGF, HB-EGF FGF7 TGF-α and TGF- β [18]. Subsequently, keratinocytes can infiltrate the blood clot by production of uPA and tPA, which induce degradation of the fibrin network via the conversion of plasminogen to plasmin. The collagen bundles are degraded by MMP-1 and MMP-9 [10]. In normal wound healing, activation of keratinocytes is maintained until reepithelialization is completed.

2.2.2. Neovascularizaton

In wound healing, increased blood flow towards the wound site is needed for sufficient oxygen delivery for the increased metabolism of the proliferating cells at the wound site. Blood vessels are formed in the granulation tissue simultaneous with the migration of fibroblasts and macrophages [19].

Endothelial cells in existing blood vessels near the wound site can release angiopoietin-2 in response to hypoxia [20] and inflammatory stress. Angiopoietin-2 competes with angiopoietin-1 for the tie-2 receptor. In this autocrine manner, angiogenesis is activated by tie2-activation in endothelial cells. VEGF possibly also plays a role in vascularization, stimulating endothelial cells to engage

sprout formation by affecting vascular permeability. Other functions of VEGF include endothelial cell migration, proliferation and survival [23]

Migrated endothelial cells degrade the blood clot by releasing tPA as described previously. It is believed that pericytes function as vascular support cells in the neovascularisation. Pericytes possibly also produce collagen [21] and can differentiate into (myo) fibroblasts [22]. In the later stages of the wound healing process, vascular regression takes place until optimal vascular density for a sufficient blood flow is reached.

2.3 Scar-remodeling phase

2.3.1. ECM maturation

It is in the scar-remodeling phase when a difference between normal scar formation and hypertrophic scar formation can first be seen. There is a difference in ECM composition between both types of scar formation, which becomes visible when the ECM matures in the scar-remodeling phase. The characteristics of the ECM in hypertrophic scars will be described later, as we will focus on ECM composition in normal scar formation in this section.

In the proliferative phase granulation tissue is formed, consisting of fibroblasts, ECM and newly formed blood vessels. A prominent component of the ECM in the granulation phase is hyaluronan (HA). HA is a glycosaminoglycan produced by fibroblasts via PDGF stimulation. It contributes to the cell migration and proliferation in the proliferative phase [24] and it also limits collagen production by fibroblasts, thereby also limiting the ECM production. In the scar-remodeling phase HA is replaced by proteoglycans. Decorin is an example of a proteoglycan incorporated in the ECM in the scar-remodeling phase. It can regulate collagen fibrillogenesis by binding to TGF-β [25], contributing to the maturation of ECM in wound healing. Collagen fibrils in the ECM of normal skin tissue are composed of collagen type I and III. Type III comprises almost 20% of the total amount of collagen. In granulation tissue the amount of collagen type III can increase up to an amount of 50% [25]. However, during scar remodeling the expression of collagen type III collagen decreases to reach normal levels. The main cells responsible for collagen production are fibroblasts and myofibroblasts.

2.3.2. Myofibroblast activation and apoptosis

When fibroblasts are differentiated into myofibroblasts, production of ECM components increases and contractile abilities are gained. By contraction of the skin, the skin defect decreases. In the proliferative phase, wound contracture is completed and the wound is fully epithelialized. In the scar remodeling phase the numbers of myofibroblasts decreases drastically. Reversal of myofibroblasts and dedifferentiation into fibroblasts is seen *in vitro* in response to a variety of factors, but has however never been demonstrated in vivo. Instead of de-differentiation, massive apoptosis takes place in the final phase *of* wound healing *in vivo* [31]. By this same mechanism inflammatory cells make place for granulation tissue formation during the proliferative phase.

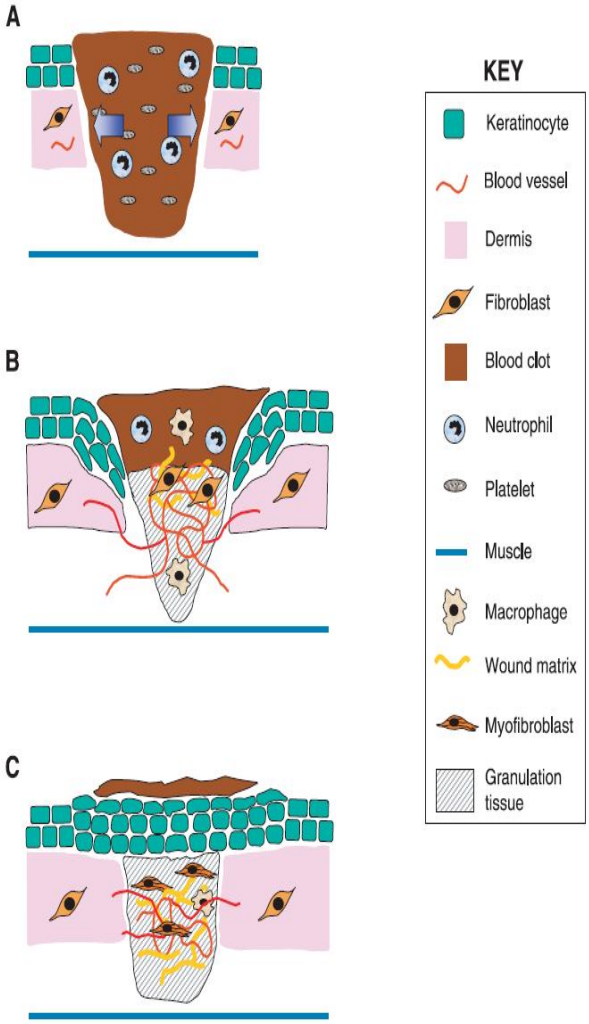

Fig. 1. Illustration taken from Werner S. and Grose R., 2003 [8]. Schematical representation of normal wound healing process at 12-24 hours after injury (A), 3-7 days after injury (B) and 1-2 week after injury(C). (A) Blood clot is formed with presence of platelets and neutrophiles. The degranulation of platelets and cytokine release of neutrophiles lead to migration of macrophages, which are present during the proliferative phase at 3-7 days after injury (B). Fibroblasts are attracted to the wound site, blood vessels are formed and keratinocytes start proliferating to create epithelium at the wound site in an intimate cross-talk with fibroblasts. Fibroblasts produce ECM and granulation tissue is formed. (C) Reepithelialization is completed, fibroblasts are differentiated into myofibroblasts, responsible for wound contraction. Scar remodeling takes place.

The signals leading to myofibroblast apoptosis remain unclear. A possible mechanism for the increased apoptosis is a relieve of stress for myofibroblasts because of a regain of original mechanical properties by the remodeled ECM. Studies have showed that by splinting the wound area and thereby preventing the tension release by granulation tissue, myofibroblast apoptosis is inhibited. However, subsequent splinting is a cause for increased cell death [32].

After the decrease in myofibroblast cells, the wound healing process is completed. The wound area is reepithelialized and the ECM composition in the granulation tissue is similar to normal skin.

3. Altered molecular and cellular mechanisms in hypertrophic scar formation

As described above, hypertrophic scar formation becomes visible in the scar remodeling phase, but the cellular and molecular causes take place early in the wound healing process. An exaggerated inflammatory response is believed to be the first step in the pathogenesis of HSF. In this section, altered mechanisms in all phases of wound healing that could give rise to HSF are discussed.

3.1 Blood clot formation

A difference between burn wounds and 'normal' wounds is that burn wounds are characterized by coagulation of superficial blood vessels and therefore no excessive bleeding takes place in burn wounds. However, the formation of a blood clot does occur in burn wounds. Where in normal wound healing the fibronectin expression in the wound stops shortly after closure of the wound, fibronectin concentration remains high for months in hypertrophic scars [71]. As described above, fibronectin is an attractant for endothelial cells and fibroblasts. As a logical result, long term high levels of fibronectin could be partly responsible for increased vascular density and increased ECM production by fibroblasts seen in hypertrophic scars.

3.2. The inflammatory phase

In hypertrophic scar formation, the inflammatory response is exaggerated. This leads to increased release of many cytokines. PDGF, IL-4 and TGF-β are cytokines that are present in significantly higher concentrations in HSF wounds and are therefore potential pro-fibrotic mediators. A common effect of the increased levels of these potential pro-fibrotic cytokines is the overstimulation of fibroblasts to produce components of the ECM.

PDGF has been described in the previous section as a growth factor released by platelets, involved in the migration of inflammatory cells. Another function of PDGF is activation of fibroblasts. In non-healing wounds PDGF was found to improve the healing process by activating fibroblast proliferation and their ECM production [33]. However, in hypertrophic scars increased levels of PDGF are found, suggesting that high levels of PDGF in later phases of wound healing have a pathogenic effect.

IL-4 is a cytokine that stimulates a Th2 response, thereby polarizing the T helper cell response. This polarized Th2 response is present in HSF and higher IL-4 concentrations are found in hypertrophic scars compared to healthy controls [34]. Besides T-cell regulation, IL-4 can stimulate collagen and fibronectin production by present fibroblasts [35]. Mast cells are the source of IL-4 in the wound area. They are interspersed with the collagen bundles and present in both normal as hypertrophic scar formation. Next to Il-4, mast cells also produce TGF-β1 and TNF-α, promoting fibroblast proliferation [36]. Mast cells also release histamine in response to pathogens. The main function of histamine is increasing the permeability of capillaries to stimulate the inflammatory response, but it can also enhance collagen production by fibroblasts [37].

One more difference between the inflammatory phase of HSF and the inflammatory phase of normal scar formation is the presence of fibrocytes, which is found to be higher in HSF [40]. The higher numbers of fibrocytes in HSF can result in more production of growth factors and ECM and ultimately higher numbers of (myo)fibroblasts, as fibrocytes are possible precursors of fibroblasts [27][28].

Finally, the cytokine TGF-β plays a major role in the wound healing process, including the inflammatory phase. There are three isotypes (TGF-β1, TGF-β2 and TGF-β3), all of which play a role in the mobilization of inflammatory cells, fibroblast infiltration and proliferation, ECM production and angiogenesis in later stages of wound healing. *In vivo,* TGF-β is secreted in a latent complex. This complex consists of the growth factor self, latency-associated peptides (LAPs) and latent TGF-β binding protein. When in this complex, the mature TGF-β cannot bind to its receptor. Release of the growth factor occurs in the process called TGF-β activation [41].

Many cells produce and/or respond to TGF-β, making it a potential target for treatment of HSF. Overexpression of TGF-β1 and TGF- β2 is associated with HSF, while the functions of TGF- β3 are mainly anti-fibrotic [38]. The possibilities of TGF- β isotype regulation as a treatment for HSF will be discussed later.

During the inflammatory phase, TGF-β is produced by platelets and functions as a chemotactant of fibroblasts and monocytes. Monocytes transform into macrophages at the wound site, and subsequently start producing cytokines, including also TGF-β. TGF-β produced by macrophages can possibly induce HSF by stimulating excess ECM production by fibroblasts [39].

3.3. The proliferative phase

3.3.1 Exaggerated processes

The proliferative phase in normal wound healing consists of wound contracture by myofibroblasts aiding reepithelialization by proliferation and migration of keratinocytes, and neovascularization of the granulation tissue. In HSF reepithelialization is prolonged, myofibroblasts activation is increased and neovascularization is augmented.

The exaggerated inflammatory response manifested by prolonged fibronectin expression in the blood clot and high concentrations of inflammatory cytokines such as PDGF, IL-4 and TGF-β is followed by exaggeration of most of the processes in the proliferative phase. The abnormal processes during the proliferative phase in HSF are described here.

3.3.2. Consequences of increased fibroblast and keratinocyte activation

In the formation of granulation tissue in normal scar formation, there is a balance between production and degradation of the ECM. This balance is disrupted in HSF as ECM degradation is decreased and ECM production by fibroblasts is increased.

There are multiple causes for the increase in ECM production by fibroblasts in HSF. First of all, (myo)fibroblast density in the granulation tissue is increased in HSF [42]. Moreover, the fibroblast activation is increased. Increased fibroblast activation and migration have been promoted by multiple cytokines in the inflammatory phase.

Also involved in both increased density and increased activation of fibroblasts are keratinocytes. In normal wound healing, keratinocytes and fibroblasts reinforce each others activity in the beginning of the proliferative phase, but down-regulate each other's activity later in the wound healing process; fibroblasts inactivate keratinocytes by TGF-β expression [43] and keratinocytes inhibit collagen production of fibroblasts by releasing cytokines [44]. Keratinocytes remain active in HSF, possibly contributing to the increased activation of fibroblasts. The exact cause of the prolonged activity of keratinocytes is not known [30].

The fibroblasts in HSF have also been found to have increased expression of CTGF. This growth factor stimulates their own chemotaxis and proliferation [45]. Moreover, CTGF is capable of regulating ECM degradation by MMP's and their inhibitors, TIMP's. In this way, CTGF is contributing to both increased ECM production and decreased ECM degradation.

Table 1. Altered mechanisms in hypertrophic scar formation

Phase	Altered mechanism/process	Effect
Inflammatory	prolonged fibronectin expression	- increased chemotaxis of endothelial cells and fibroblasts - more ECM production and neovascularization in later phases
	increased PDGF release by platelets	- increased chemotaxis inflammatory cells - increased ECM production by fibroblasts
	increased IL-4 release by mast cells	- polarized Th2 response - increased collagen and fibronectin production by fibroblasts
	increased histamine release by mast cells	- increased collagen production by fibroblasts
	increased TGF-β release by platelets and consequently by macrophages	- increased chemotaxis fibroblasts and monocytes - increased ECM production by fibroblasts
	higher number of fibrocytes	- increased density of fibroblasts - Increased ECM production
Proliferative	prolonged activity of keratinocytes	- increased fibroblast activity
	increased CTGF release by fibroblasts	- increased chemotaxis fibroblasts - increased fibroblast proliferation - decreased ECM degradation (MMP and TIMP regulation)
	lower oxygen concentration in burn wounds	- increased angiopoietin-2 leading to increased neovascularization
	increased VEGF serum levels	- possibly leads to increased neovascularization
Scar-remodeling	increased collagen III production by fibroblasts	- altered fibril structure
	decreased decorin production by fibroblasts	- increased proliferation of fibroblasts - increased collagen and TGF-β production by fibroblasts
	more bone-like cross-links in fibrils after LH2b production by myofibroblasts after TGF-β stimulation	- decreased susceptibility of the ECM to degradation
	increased TIMP production and decreased MMP production by HSF myofibroblasts	- decreased degradation of the ECM
	decreased apoptosis of HSF myofibroblasts (?)	- contractures of the wound and prolonged and increased ECM production

3.3.3. Excess blood vessel formation

Factors that possibly play a role in the excess blood vessel formation in HSF are the reduced oxygen levels present in burn wounds [46] and conditions in favor of angiogenic sprouting created by the exaggerated inflammatory response.

High levels of VEGF are found in the serum of burn victims [47], suggesting that increased VEGF expression plays a role in augmented vascularization. The exact underlying mechanisms of augmented vascularization in hypertrophic scar formation are not yet understood.

3.4 Scar-remodeling/ resolution phase

3.4.1. ECM maturation

In the final phase of hypertrophic scar formation, the red, raised skin, rough texture and stiffness described in the introduction are seen. These characteristics are manifested as a consequence of the excess amounts of ECM in hypertrophic scars and most importantly the abnormal maturation of this excess ECM. The normalization of collagen ratios as seen in normal scar formation is not present in the scar-remodeling phase of HSF, as collagen type III expression remains high during this phase [48]. High ratios of collagen III results in a decreased fibril diameter, as shown in self-assembly *in vitro* experiments [49].

Hyaluronan is less replaced by decorin in HSF compared to normal scar formation, since hypertrophic scar fibroblasts produce less decorin [50]. Therefore, the suppressive effect of decorin on cell proliferation and collagen I and TGF-β production is less present in HSF.

Bone-like cross-links are established in fibrils of hypertrophic scar ECM, mediated by telopeptide lysyl hydroxylase (LH2b) produced by myofibroblasts under influence of TGF-β. These bone-like crosslink make the ECM less susceptible to degradation by MMP-1[51]. Next to this lesser susceptibility to degradation, hypertrophic scar myofibroblasts express less MMPs and more TIMPs [52], contributing to abundant ECM in the remodeling phase.

3.4.2. Myofibroblast activation and apoptosis

Burn wounds of burn victims often are characterized by contractures, which are probably caused by the high amount and activation of myofibroblasts. It is believed that the induced apoptosis of myofibroblasts, which is responsible for the regulation of wound contraction and scar remodeling, is reduced in HSF [53]. The mechanisms that cause this reduced apoptosis are unclear. Some studies are contradictory on the amount of apoptosis present in hypertrophic scar formation, as not always reduced apoptosis is measured and in some studies even an increase in apoptosis is found [54].

3.5 Overview of altered mechanisms in hypertrophic scar formation

In table 1, all the altered mechanisms in HSF that have been discussed in this section are summarized, categorized by phase. The underlying principle is that the first step towards HSF is an exaggerated inflammatory response, leading to derailed processes during the proliferative phase. TGF-β plays a very important role in many of these derailed processes, and the isotypes TGF- β1 and TGF- β2 have been found to be pro-fibrotic. The regulation of the isotypes of this cytokine is believed to have great potential in the prevention of hypertrophic scar formation. Finally, in the scar-remodeling, the characteristics of HSF become tangible as a result of abundant and abnormally composed ECM. At the site of injury a mass of non-functional tissue is formed.

4. Current research and future preventive treatments of hypertrophic scar formation

The most commonly used current treatments of hypertrophic scar formation include occlusive dressings, topical silicone gels and sheets, pressure garments, radiation, intralesional corticosteroid injections, cryotherapy, surgical revision and laser revision. All of these therapies are quite burdensome for the patient, and the positive effects are not optimal.

Surgical revision is a very common used therapy for hypertrophic scars. The area of the wound is reduced and the tension of the wound is regulated. However, recurrence of hypertrophic scars is seen. To decrease the chance of recurrence, surgical intervention should be used in combination with radiation therapy, silicone gel or sheets or intralesional corticosteroid injection. In HSF, corticosteroid injections are very painful and this therapy is used for keloids rather than for HSF. The mechanism of action of both silicone and corticosteroid injections are not well understood and the results on HSF are quite variable. The combination of surgical revision and radiotherapy is also successful, but there is some concern over the occurrence of post radiation cancer. It is suggested that radiation should only be used as a treatment for HSF when other treatments are ineffective.

Cryotherapy and laser therapy are both therapies aiming at reducing tissue bulk. Necrosis and apoptosis is induced, and as a result, the scars are less raised. Laser therapy is however not very effective and cryotherapy is associated with many side effects, including pain, oedema, wound infections, etc.

4.1. Possible targets for preventive treatments

Overall, the results of current treatments for HSF are modest. In view of the altered mechanisms described in the

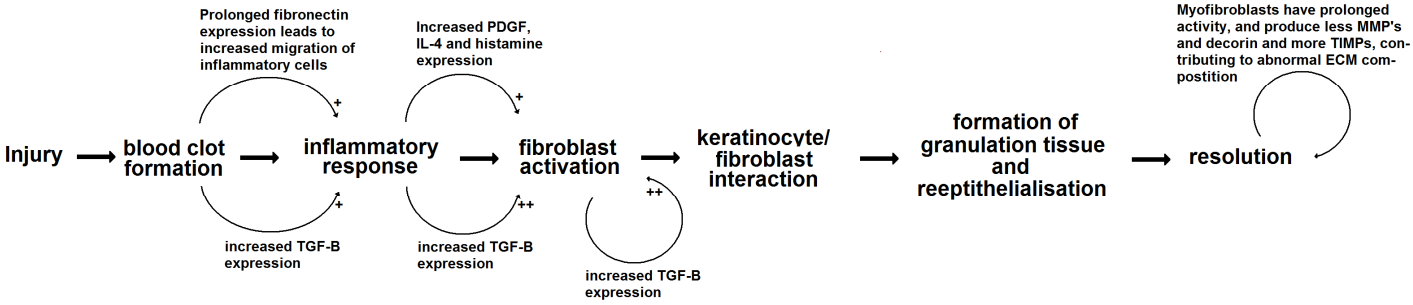

Fig. 2. Abnormal processes and potential targets of therapies against hypertrophic scar formation. The cascade of steps in hypertrophic scar formation is the same as in 'normal' wound healing. The main abnormal processes that outbalance this cascade are outlined above. These abnormal processes can be categorized in three groups, providing three potential targets; inflammatory mediators, TGF-β and prolonged myofibroblast activation.

previous section (see also table 1), several preventive therapies for HSF can be proposed. Figure 2 gives an overview of abnormal processes and mechanisms in HSF, not focusing on the effects and results of these processes. The three categories in which they can be divided are inflammatory mediators, TGF-β expression and prolonged myofibroblast activation. These categories can all serve as potential targets of HSF.

Targeting inflammatory cytokines and thereby preventing the exaggeration of inflammation as seen in HSF would be the most effective as a preventive treatment. Suppression and regulation of the inflammatory response could eventually abrogate excessive fibroblast activation and ECM production. Also, an abnormal inflammatory response is responsible for outbalancing the healing process in a very early stage. Targeting early steps would be most effective to bring the healing process back on track.

TGF-β expression is also a powerful target that will be discussed later in this section. Because TGF-β plays many roles in many stages of wound healing, modulating TGF-β expression can be a very effective therapy for preventing hypertrophic scar formation.

A more difficult target is the prolonged myofibroblast activation. Early in the wound healing process myofibroblast play an essential role. However, in the final stage, myofibroblasts contribute greatly to an abnormal ECM composition and wound contraction.

For all of the potential targets it should be kept in mind that both normal and abnormal wound healing are processes regulated in time and space. Many mediators and cells that play an essential role in one stage in wound healing, can have a negative effect in other stages. Therefore, completely knocking out mediators as a therapy for HSF will always have side-effects.

In the next section, current research targeting inflammatory mediators, TGF-β expression and also the targeting of fibroblasts and myofibroblasts are separately discussed.

4.2. Targeting inflammatory mediators in hypertrophic scar formation

The delivery of exogenous cytokines and growth factors in the wound healing process has been extensively studied. For example, accelerated reepithelialization has been observed in burn patients when exogenous EGF was added to the wound site [55]. In order for addition of exogenous EGF to be effective, the EGF receptor must be expressed by target cells, in this case by keratinocytes present at the wound site. In the first section it is described how in normal wound healing keratinocyte migration towards the blood clot is stimulated by EGF.

In several studies, exogenous cytokine or growth factor addition was unsuccessful due to enzymes at the site of injury, a lack of adequate receptors and also due to possible side effects occurring when sufficient amounts are added to the wound. Therefore, gene therapy seems to be a better and more effective method for cytokine and growth factor therapies.

Efficient transfection of genes in cutaneous wounds has shown to be difficult to establish. For optimal gene therapy, the gene must be accepted by the targeted cells, and moreover the gene must be expressed at sufficient levels for the right amount of time [56].

The use of several delivery mechanisms and vectors has been studied. In cutaneous wounds, viral transfection is very ineffective and potentially dangerous, considering toxicity and immunologic comprise [57]. Delivery of naked non-encapsulated DNA or plasmid constructs topically or by a pneumatic gene gun also have low transfection efficiencies. The use of non-viral liposomal cDNA genes is a more promising method. The use of cytomegalovirus promoters in the cDNA together with modification of the liposomal structure to a cationic structure and the inclusion of cholesterol increases transfection efficiency and transgenic expression levels [58]. Another approach is the use of bio-degradable polymers incorporated with a

therapeutic gene, which is a promising delivery mechanism for treatments for wound healing [59].

Even though gene therapy is more effective than exogenous addition of mediators to the wound site, it is still difficult to establish. Much more research needs to be done before gene therapy in wound healing can be successfully performed in humans. Presently, the majority of clinical trials with burn patients aiming for an improved healing process and reduced scarring consist of the addition of exogenous mediators.

4.3.3. Possible anti-fibrotic functions of Il-10.

Hypertrophic scars are associated with a polarized Th2 response. In the previous section IL-4 production by Th2 cells has been described. Other cytokines produced by Th2 cells are IL-5, IL-6, IL-10, IL-13 and IL-21. Increased serum levels of IL-4, IL-6 and IL-10 are found in HSF patients [34]. Interestingly, IL-10 is found to have anti-fibrotic functions. IL-10 reduces the expression of IL-6 and IL-8, which both are pro-inflammatory cytokines [66]. These findings contradict the theory that the polarized Th2 response in hypertrophic scar formation is simply a fibrotic response.

Injection of recombinant IL-10 in the margins of acute incisional wounds results in reduced scarring. Addition of IL-10 as a preventive treatment for excessive scarring is currently evaluated in a single-centre, double-blind, placebo controlled, randomized phase II efficacy trial to include <1400 wounds. Subsequent clinical trials for the prevention of scarring associated with peripheral nerve damage are planned if results remain positive.

4.3. Targeting TGF-β expression in hypertrophic scar formation

TGF-β is one of the major inflammatory mediators involved in HSF. TGF-β1 and TGF-β2 expression is increased in hypertrophic scar formation. However, the TGF-β3 isotype functions as an anti-fibrotic mediator. Also, increased TGF-β3 expression is observed in scar free embryonic wound healing. Scar formation during wound healing is a process which occurs in adult mammals and also during the last trimester of gestation in mammals. Interestingly, until halfway gestation time, perfect scar-free healing is observed [60]. The collagen composition of the regenerated tissue resembles the collagen composition of normal skin, unlike the connective tissue in normal scars and especially in hypertrophic scars.

Scar free embryonic healing was first discovered when sheep embryo's where investigated as surgical models for cleft lip. In these embryos scar free healing of the lip was observed, leading to further investigation of differences between adult scar-forming healing and embryonic scar-free healing, and the cellular and molecular mechanisms that are involved. Differences in TGF-β isotype expression were found, as in adult scar-forming healing TGF-β1 and TGF-β2 expression is high and TGF-β3 expression is low, whereas in embryonic scar-free healing TGF- β1 and TGF-β2 expression is low and TGF- β3 expression is high.

Deletion (knock-out) of TGF-β3 in embryonic transgenic animals results in delayed wound healing and scar formation, compared to littermates in which both copies of TGF-β3 are present. Decreased epithelialization and dermal cellularity is seen in TGF-β3 deficient embryonic animals. Follow-up studies demonstrated that TGF-β3 plays a role in the migration of keratinocytes and other dermal cells. Both the increased levels of TGF-β observed in HSF and the evidence that expression of different TGF-β isotypes plays an important role in scar-free embryonic healing suggest that regulation of TGF-β expression in HSF is a promising preventive treatment.

4.3.1. TGF-β isotype regulation

In 1994 and 1995, Shah et al demonstrated that regulation of TGF-β isotypes reduced scar formation in rodents and rats. More specifically, neutralization of TGF- β1 and TGF-β2 by the addition of antibodies or addition of exogenous TGF-β3 reduced scar formation after induction of cutaneous incisional wounds. Neutralization of both TGF-β1 and TGF-β2 resulted in reduced monocyte and macrophage profile, neovascularization, collagen I and III deposition in early stages of wound healing, and most importantly a markedly improved architecture of the neodermis was observed. Neutralization of only one of these two isotypes had less effect. Exogenous addition of TGF- β 3 had the same effect as neutralization of TGF- β1 and 2, suggesting that TGF-β1 and 2 neutralization possibly leads to increased amounts of TGF-β3 in the wound [61] [62].

When significant molecular comparability between rat models and humans was proven, clinical trials of exogenous TGF-β3 addition in humans were started by Occleston et al. In these double blind, randomized, within subject placebo and standard care controlled phase-I and -II studies in human volunteers, the appearance of scars was evaluated after injection of exogenous human TGF-β3 in full thickness cutaneous incisional and excisional wounds. Evaluation of the appearance of the scars show reduced scar formation after treatment with TGF-β3 in a statistically significant majority of 70%. Moreover, frequency and optimal dosage levels could directly be translated from pre-clinical studies in rats to dosage and frequency in man, indicating great similarity between the species and the good utility of rat models in research on the healing process [63].

The effect of exogenous TGF-β3 addition on scar formation in humans, as for any other treatment, can only be evaluated by the visual assessment of scars. For the

understanding of possible underlying mechanisms there must be relied on observations in pre-clinical studies in rats, and on the molecular similarities proven between the healing processes in rats and man.

4.3.2. Other strategies in TGF-β regulation

Besides TGF-β targeting by exogenous addition of TGF- β3, a clinical trial is being done on exogenous addition of sterile mannose-6 phosphate. Mannose-6 phosphate competitively inhibits the latency associated protein incorporated in the TGF-β latency complex, thereby preventing TGF-β activation [64]. The goal of this treatment is the inhibition of the high TGF-β1 expression in scarring. Hereby regulating TGF-β isotype expression, scar formation can be reduced. Also, there has been pre-clinical success in reducing scar formation by the addition of decorin [65].

Another approach in the regulation of TGF-β activity is targeting the TGF-β signaling pathway. After binding to trans-membrane receptors, TGF-β activate intracellular regulatory proteins called Smads. Smads subsequently activate target genes including procollagen I and III [67]. Smad 3 signaling is associated with fibrosis [68]. Smad 7 on the other hand inhibits smad3 signaling *in vitro* [69]. Increasing SMAD7 expression by pharmacologic treatment or gene therapy and thereby inhibiting pro-fibrotic functions of TGF-β could be a potential preventive treatment in HSF. The construction and expression of a recombinant human smad7 adenoviral vector and has already been established *in vitro* [70], but more research needs to be done.

4.4. Targeting (myo)fibroblasts in hypertrophic scar formation

4.4.1. Collagen synthesis

Excessive ECM production and abnormal ECM composition by both fibroblasts and myofibroblasts are the major result of pathophysiological processes in HSF. Therefore, ECM production is an obvious target in the prevention of excessive scarring. This approach is focused on decreasing the symptoms of HSF rather than preventing the development of HSF. The strategies which are being investigated focus on the pharmacological modulation of collagen synthesis.

4.4.2. Inhibitors of prolyl 4-hydroxylase and tissue transgutaminase

Two collagen synthetic reactions that have been targeted are the intracellular hydroxylation of proline residues and the enzymatic cross-linking of procollagen molecules[73].

The hydroxylation of proline residues is necessary for the formation of procollagen bundles and is catalyzed by prolyl 4-hydroxylase. Several inhibitors of prolyl 4-hydroxylase have been found. Decreased collagen deposition was found in a rabbit ear model of HSF after application of topically active FG-14648. Doxorubicin is a commonly used chemotherapeutic agent also known to inhibit collagen synthesis by irreversibly inactivating prolyl 4-hydroxylase. The results of doxorubicin therapy are not optimal however, but both general decreased protein synthesis and decreased fibroblast proliferation have been shown.

Previously has been described how collagen III expression remains high in HSF scars, in comparison with normal scarring, where collagen III expression decreases in the scar-remodeling phase to reach normal skin tissue levels. Inhibition of procollagen II cross-linking might result in improved ECM composition and decreased ECM deposition in HSF. The cross-linking of type III procollagen is mediated by the enzyme tissue transglutaminase, according to literature studying acute wound tissue explants in rats. An inhibitor of tissue transglutaminase is putrescine. In phase II clinical trials of this inhibitor the scar ratings were uniformly better than in untreated wounds. The effect of putrescine was dependent of the severity of the wound. In severe wounds, the best results were seen, but in less severe wounds the results were more ambiguous. The easy application of putrescine as a topical agent and the minimal side effects seen in the clinical trials make it a promising treatment in HSF.

In some studies, the use of antisense oligo-deoxynucleotides is investigated *in vitro*. For instance, Col A1 antisense oligo-DNA treatment of *in vitro* human fibroblasts derived from HSF scar tissue results in decreased collagen I A1 mRNA and protein expression. The use of cationic liposomes show increased efficiency. Antisense oligo-DNA as a treatment in HSF is however still in its infancy.

4.4.4. Suppressing fibroblast differentiation into myofibroblasts

In a recent study on the effect of human decorin transfection in a human corneal fibroblast cell culture, less α-SMA expression was observed[72]. This suggests that the transformation of fibroblasts into myofibroblasts is suppressed by decorin transfection. As described earlier, decorin is known to have an inhibitive effect on TGF-β, resulting in a decreased collagen synthesis and also decreased fibronectin expression by fibroblasts. In the future there are possibilities to study the effect of human decorin transfection at the wound site in burn patients. In normal wound healing, decorin is expressed, but in HSF patients, less decorin expression by fibroblasts has been found. If decorin is transfected in the later stages of HSF, the negative effect of high myofibroblast activation can be prevented.

5. Conclusion

Hypertrophic scar formation is the result of many pathophysiological processes, and many altered mechanisms are found in wound healing in HSF patients compared to normal wound healing. The most knowledge of the pathogenesis of HSF is established by combining observations in simple *in vitro* experiments, animal models and observations in human patients.

The complexity of HSF is very difficult to mimic *in vitro*. Animal models provide a better source, but currently there is a lack of standardized animal models or HSF. Circular excisional wounds in rabbit ears are known to produce a temporary hypertrophic wound. Also the red duroc pig has scarring capabilities, but long term research in this model is very expensive. Most HSF wounds in animal models are induced by incisional or excisional wounds. The limiting factor in observations in human HSF patients is the lack of controlled wounds. Also, HSF processes are not uniform in patients, because of differences in the size of the wound, the location of the wound and the age of the patient.

The limits in *in vivo* research, animal models and human patients make it very difficult to investigate pathophysiological processes in HSF. Of many altered mechanisms in HSF, it is unknown if they are a cause or a consequence of hypertrophic scarring. Much more research needs to be done before the etiology of HSF is fully understood. Only when the etiology of HSF is completely elucidated, effective preventive treatments can be found. Research on other forms of excessive scarring, such as keloids, but also haze formation in the cornea and fibrosis in internal organs can provide new insight and strategies for preventive treatments of HSF.

Current treatments of HSF are inefficient and burdensome for the patients. Also, the results are variable and often modest. Research on the etiology of HSF has lead to the development of new preventive treatments.

Three strategies can be used in preventive therapies of HSF; down-regulation of inflammatory mediators, TGF-β isotype regulation and modulation of (myo)fibroblast activity. Targeting collagen production by both fibroblasts and myofibroblasts reduces the symptoms of HSF by a reducing excessive ECM deposition. However, the cause of HSF; the underlying mechanisms causing excessive ECM production, are not affected by this therapy. Modulating myofibroblast activity by preventing the differentiation of fibroblasts will also result in reduced symptoms, but again the mechanisms that are the cause of excessive scarring are not affected. In this regard, the targeting of inflammatory mediators is potentially a better preventive therapy for HSF. By targeting inflammatory mediators early processes in HSF are affected. However, because many cells produce and respond to the inflammatory mediators present at the wound site, side effects are to be expected.

Much research has been done on the possibilities of TGF-β regulation. By down-regulating TGF-β1 and TGF-β2 and/or up-regulating TGF-β3, scar formation is significantly decreased and negative side effects are minimal. By this regulation of TGF-β isotypes, embryonic expression levels are imitated. The goal is to achieve scar-free wound healing, as seen in the first half of gestation time in embryos. Further research on TGF-β isotype regulation is warranted to lead to great improvement of the wound healing process in HSF patients. Gene therapy might be a promising approach in the use of TGF-β isotype regulation in preventive therapies for HSF.

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