Hypertrophic Scars after Burn Injury

Restoration to Scarless Skin?

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Abstract

50% of the victims of burn injury develop hypertrophic scars. A hypertrophic scar is a raised, reddish scar, which is caused by a delayed wound healing process which causes an excessive deposition of extracellular matrix. This scar tissue can cause physical and psychological harm to the victim. For the prevention of hypertrophic scar formation, the pathophysiology of the wound healing process leading to this scar tissue has to be known.

Wound healing is a multistep process with a lot of different cells and mediators involved. This process can fail in many phases causing the formation of an hypertrophic scar. This can be caused by an increased immune response, decreased apoptosis of cells and an increased deposition of extracellular matrix. Inhibition of single cell mediated processes of the wound healing mechanism, can slowly turn the wound healing process of hypertrophic scar tissue back to the normal wound healing process, by reducing the exaggerated inflammation and the collagen deposition. The main targets to alter are the excessive TGF-β secretion, the Th2-lymfocyte response and the inhibition of matrix metalloproteinases. Alteration of these mechanisms will not lead to completely scarless wound healing, but it may reduce the risk of hypertrophic scar formation. Fetal wound healing shows us that scarless wound healing is a possibility, however to reach this goal, a lot of research still has to be done.

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Introduction

Trauma is one of the leading causes of death and disability, the second most common causes of death due to trauma is burn wound trauma. When a patient survives burn trauma's there are still many problems occurring. Thermal injuries have a major impact on the life of the victims. There are questions about the restoration of the scar tissue formed to the state before the injury, the functionality of the harmed tissue, and the psychological harm. *(Goel et al, 2010)*

All wounds in the adult body will heal with a scar, but the level of inflammation will determine the impact on the final scar formation. There are a lot of different forms of scar tissue, all formed as a result of wound healing. Research showed that shallow dermal burn wounds, which heal within ten days, normally don't develop scar tissue. The longer the burn wound takes to heal, the risk of scarring increases. Besides shallow dermal scars, burn scars are inevitable, but it depends on a lot of factors. These factors differ from sex, age and ethnic origin to the depth and location of the burn injury. *(Hall et al, 2011)* However, when burn wound healing fails, it can lead to a fibrotic form of post burn scars. *(van der Veer et al, 2008)*

This scar tissue is one of the most common complications after thermal injury, and is called hypertrophic scars. Hypertrophic scar tissue can also be formed after trauma, inflammation, surgery, or even spontaneously. However, it occurs in almost 50% of the burn victims with deep dermal wounds. This firm and inflammated scar tissue stays in the area of the primary burn injury and are elevated above the skin surface. The prominent and visible scars can lead to a decrease of body image and ability to function of the victim. *(Hall et al, 2011)*

Studies showed huge differences between adult wound healing and fetal wound healing. Embryonic wounds heal perfectly, with little or no scar formation. In both wound healing processes, extracellular matrix contractions closed the wounds. In the fetal wound tissue, TGF-β levels are decreased, there is no conversion from fibroblasts to myofibroblasts and there is a decreased inflammation reaction. When fetal wounds were treated with TGF-β, scars did occur. These findings show that scarring is a by-product, and in the right environment scarless wound healing can occur. *(Penn et al, 2012)(Martin, 1997)*

For prevention, management, restoration or treatment of burn wound injury, you have to understand the mechanism of burn wound healing. Which factors causes the formation of hypertrophic scar tissue, where the wound healing mechanism fails and is there a way to inhibit this mechanism and cause restoration of the pre-injury state?

Hypertrophic Scar Tissue

The skin is the largest organ in the body and has multiple functions. It is a protective barrier and prevents dehydration, protects the skin against UV radiation and plays an role in keeping the body temperature at a normal level. Besides the protective role, the skin also has a role in the social appearance. When the skin is burned, the victim will not only have physical problems, the patient will also encounter the psychological effects. Burn wounds cause a loss of epidermal integrity and the process of wound healing is necessary to restore the integrity of the skin barrier. The most common complication after burn injury is abnormal scarring. *(Bombarro et al, Burns, 2003)*

The skin consists of 4 major layers, the epidermis, the basement membrane, the dermis and the sub cutis. The epidermis is the outer layer of the skin and the first immune barrier of the body. The major cell type in this layer is the keratinocyte, or epithelial cell. In normal burn wound healing, when this layer is harmed, a scar is formed, but the scar will not thicken and regresses soon. The dermal layer can be divided in two layers, a thinner papillary layer and a thicker reticular layer. The papillary layer forms the connection with the epidermis and the collagen fibers lie loosely, as the reticular layer, which contains thick collagen fibers, a lot of extracellular matrix producing fibroblasts, macrophages and mast cells, extends to the subcutis layer. *(Aromadermatology, Chapter 1, 2006)*

When the dermal region, and especially the reticular layer is harmed by burn injury, scars are formed and very often scars which are characterized by an excessive deposition of collagen. There are two major forms of these scars: keloid scar tissue and hypertrophic scar tissue. They differ in collagen density, orientation and vascularity of the wound, but the main difference is that hypertrophic scars stay between the boundaries of the initial wound while keloids extent with their overgrowth of fibrosis. *(Aarabi S et al, 2007) (Meenakshi et al, 2005)* Hypertrophic scars occur in about 50% of the healed burn wounds due to dermal injury. The formation of hypertrophic scars depend on several factors, amongst them are sex, age, depth of burn, wound size and type of injury. In the following section I am going to focus on the formation of hypertrophic scars due to burn wounds, what causes them, and if there are ways to prevent the formation of hypertrophic scars.

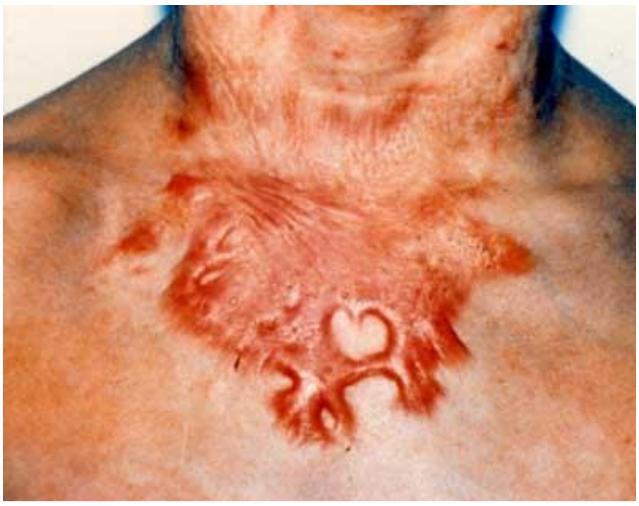

Figure 1. Hypertrophic Scar after Burn Injury

Characteristics of Hypertrophic Scars

When wound healing fails, hypertrophic scars can be formed *(figure 1).* Hypertrophic scars are a form of dermal fibrosis. There are alterations in the wound healing process which leads to the increased collagen deposition. *(Meenakshi et al, 2005)* The scars are positioned within the boundaries of the initial injury and are formed after one till three months after the initial burning. They are characterized by their stiff and rough texture, itching or feeling of pain and their red or purple color. They raise above the skin surface and often occur around joints, were there is frequent mobility. When hypertrophic scars are formed around the joints, the skin is firmer and less elastic than before, which can cause limitations in movement. This deformity around the joints, called contractures, can cause

difficulties in bending the joint and can cause pain. After a few years, the scar slowly regresses and resolves into a soft and thin scar. Hypertrophic scar formation is a fibrotic disorder, there are abnormalities in the deposition of extracellular matrix and is often paired with an immune reaction. The balance between synthesis and degradation of collagen is not in equilibrium. Due to an increased production, deposition and contraction of extracellular matrix the normal wound healing process fails, and scars are formed. One of the main compounds of extracellular matrix is collagen. *(Meenakshi et al, 2005)*

Collagen Synthesis

Collagen is the main protein in extracellular matrix. There are 28 different types of collagen, which differ length, assembly and cross-links. Collagen is produced by different cell types, including mesenchymal cells (fibroblasts, osteoblasts, odontoblasts), epithelial cells, endothelial cells, muscle cells and Schwann cells. It forms 30% of the dry weight of the body. The most abundant form of collagen in scar tissue is collagen type I and type III, these collagen types are synthesized by fibroblasts. *(Diegelmann, 2001)(Sandhu et al, 2012)*

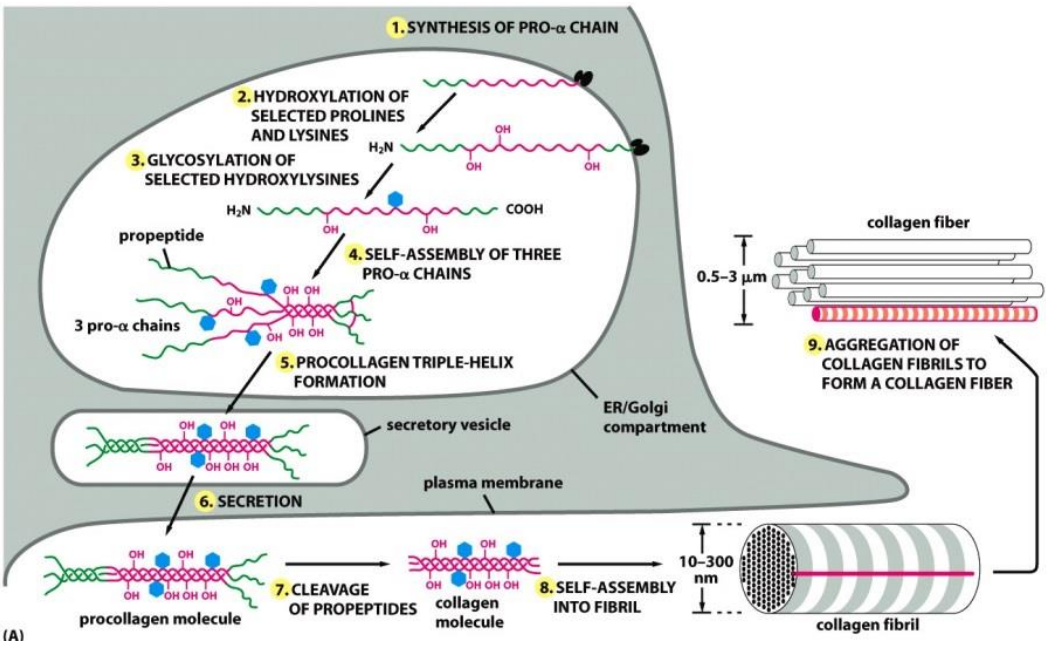

Figure 2. Collagen Synthesis Pathway in a Fibroblast Molecular Biology of the Cell, Third Edition, Alberts

The collagen synthesis *(figure 2)* is an intra- and extracellular process. At first, collagen mRNA is translated in the nucleus and a pre-pro-collagen molecule is formed. This pre-pro-collagen is trans located to the endoplasmic reticulum (ER) where the pre-domain is cleaved. A pro-collagen molecule is formed, called the α-chain. *(van Marion, 2006)*
In the pro-collagen α-chain there is a repetition of Gly-Xaa-Yaa, with proline and hydroxy proline the most abundant amino acids on the Xaa and Yaa place. After the synthesis of α-chains, proline can be modified into lysine, hydroxylysine or hydroxy proline. For the conversion of proline into hydroxy proline, vitamin C is necessary. *(Bachmann et al, 2005)*

If three α-chains are properly aligned, the Gly-Xaa-Yaa repetitions can be hydroxylatd and can form hydrogen and disulfide bonds, forming triple helices. *(Bachmann et al, 2005)* Collagen Type I is an homotrimer, and consist of the three of the same α-chains, however other types of collagen can form

triple helices with different α-chains. The ER packages the triple helices in secretory vesicles and they are transported to the Golgi apparatus and secreted into the extracellular space. *(van Marion, 2006)*

At the N- and C-terminus of the triple helices are non-helical extension proteins, which are called telopeptides, and non-helical pro-peptides. These pro-peptides make the pro-collagen soluble so it is easily transported in the cell. At the telopeptide site, from lysine or hydroxy lysine residues cross-links with collagen and other molecules can be formed. These cross-links give the collagen mechanical integrity and stability. *(Sandhu et al, 2012)*
Cross-linking happens when the lysine residues in the telopeptides are oxidized by Lysyl oxidase (LOX) into lysine-aldehyde, the lysine-aldehyde then can form cross-links, resulting in collagen. *(Sandhu et al, 2012)(Siegel et al, 1970)*However, lysine can also be converted in hydroxy-lysine by the enzyme Lysyl Hydroxylase (LH). From the hydroxy-lysine residues in the telopeptide pyridinoline cross-links are formed, which are very stable. *(van Marion, 2006)*

Shortly after the secretion to the extracellular space, N- and C-proteinases cleave of the pro-peptides, which turns the soluble pro-collagen molecule into a non-soluble collagen molecule. In a self-assembling process, the triple helices line up and can be connected via cross-links forming collagen fibrils, this process is called fibrillogenesis. Five collagen molecules together form a fibril, and the fibrils can associate into fibers. The more collagen molecules together, the stronger the fiber is. *(Diegelmann, 2001)*

After burn injury, extracellular matrix is formed in the healing process. The scar collagen formed in this process, will never regain the organized structure it had before. This is why scar collagen contains maximal 80% of the strength of the original. *(Diegelmann, 2001)*

Fibroblasts in Hypertrophic Scars

The major cell types playing a role in the hypertrophic scar formation are the extracellular matrix producing cells, the dermal fibroblasts. The fibroblast is an offspring of the mesenchymal cells and can appear throughout the body in different morphologies. The fibroblasts found in the skin are an essential element of the skin physiology. They give formation to the extracellular matrix and they play an important role in cell-cell communication via paracrine and autocrien secretion of cytokines and growth factors. *(Sorrell et al, 2004)* The interaction between keratinocytes and fibroblasts is important in the wound healing process. Keratinocytes stimulate dermal fibroblasts which in turn modulate the activity of keratinocytes. *(Werner et al, 2007)*

Many abnormalities in wound healing, are caused by a change in the function of the (myo)fibroblasts. In hypertrophic scars, there is an imbalance between the synthesis and degradation of the extracellular matrix. The fibroblasts during this imbalance have an altered phenotype, which favors the synthesis side of extracellular matrix remodeling. There is an increased density of fibroblast, elevated production of fibronectin and Type I and II collagen, fibroblasts secrete more TGF-β, versican and biglycan. On the degradation side, the activity and production of MMPs is decreased, the cells do not go in apoptosis and secrete less decorin. *(Sorrell et al, 2004)(Wang et al, 2008)*

Fibroblast to Myofibroblast Transition

Fibroblasts follow three steps in normal wound healing. At first, fibroblast migrate to the wound matrix where they secrete proteases, extracellular matrix, growth factors and cytokines. Secondly, the fibroblasts are conversed into myofibroblasts *(figure 3)*, which contain α-Smooth Muscle Actin (α-SMA) and produce, deposit and remodels extracellular matrix and stimulates wound closure. At last, after wound closure, the myofibroblasts disappear by apoptosis. *(McDougal et al, 2006)*

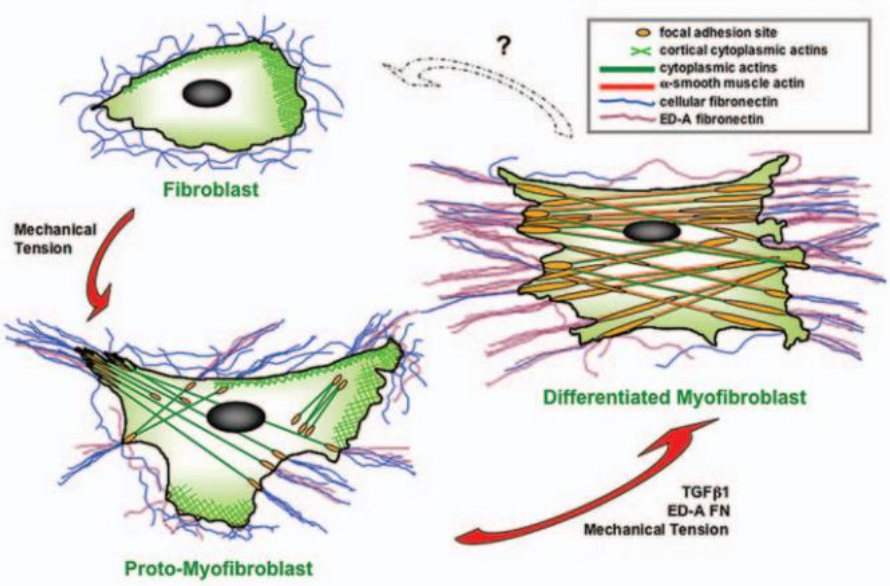

Figure 3. The Fibroblast to Myofibroblast Transition and its Mediators

The fibroblast-myofibroblast conversion can be stimulated in two ways, by mechanical tension and by TGF-β stimulation. In contrary with normal wound healing, in hypertrophic scarring, the myofibroblast persist after wound closure. This is a reason of the accumulation of extracellular

matrix and can cause increased wound contraction. It is thought that the urokinase plasminogen activator pathway (uPA) plays a role in the transition of fibroblasts into myofibroblasts. uPA is an extracellular serine protease which can bind his receptor, uPAR. This binding leads to the conversion from plasminogen to plasmin at the cell-extracellular matrix boundary. Plasmin can cleave fibrin and other extracellular matrix proteins, but it can also promote cell migration and cell adhesion by the activation and secretion of MMPs and growth factors. The uPAR on the cell membrane of the fibroblast can interact with integrines on other cells or on extracellular matrix. This leads to a reorganization and migration of the cytoskeleton. The increased cell-extracellular matrix adhesion, creates mechanical tension which is needed to put together the α-SMA, and leads to the transition to myofibroblasts. *(van der Veer, 2008)(Martin, 1997)*

First, the proto-myofibroblast is formed. This cell contains stress fibers with cytoplasmic-β and ɣ-actin. Only mechanical tension is needed to trigger the transition of fibroblasts to proto-myofibroblasts. Second, a differentiated Myofibroblast is formed, characterized by α-smooth muscle actin (α-SMA). *(figure 3)* For this transition, a mechanical trigger and TGF-β is needed. TGF-β can promote the transition by stimulating the synthesis of collagen type I and α-SMA. *(Desmoulliere et al, 2006)*
Fibroblasts can be turned into myofibroblasts, but there is no stimuli known which can stimulate the transition of myofibroblasts into fibroblasts.

Mechanical Tension
Mechanical stimuli are transduced via integrin-extracellular matrix interactions and focal adhesion kinases (FAK) and results in the cellular response from the fibroblast. Integrines are on the cell membrane and are bound intracellular with the cytoskeleton and can bind extracellular matrix with their extracellular region. In this way, integrines can transduce mechanical signals*(Margadant et al, 2010)* Studies has shown that mechanical forces have an influence on several processes of the fibroblast, amongst them differentiation, proliferation, survival, migration, fibroblast to Myofibroblast transition and matrix production. In experiments, when the mechanical tension is increased during wound healing, there is an increase in the proliferative phase, more collagen deposition was noticed and it finally resulted in the formation of an hypertrophic scar. This shows us that the mechanical environment during wound healing can also influence the formation of an hypertrophic scar. *(Aarabi et al, 2007)(Aarabi, Longaker, 2007)*

The Wound Healing Process

Wound healing consist of the four following phases: hemostasis, inflammation, proliferation and matrix remodeling. In normal wound healing, there is a balance in the anabolic and catabolic state of wound healing, were all the four phases are in equilibrium. *(Van der Veer, 2008)*

The wound healing process leading to hypertrophic scars is abnormal in comparison with normal wound healing. Especially the increased activity of the inflammation phase is one of the leading factors which causes the formation of hypertrophic scar tissue. Besides the excessive immune reaction, there is an increased production of extracellular matrix and angiogenesis, the hemostasis and remodeling phase is altered, there is prolonged re-epithelialization and decreased apoptosis of myofibroblasts. These are several ways in which the wound healing can disturbed. This can occur in all four different phases of wound healing *(figure 4)*.

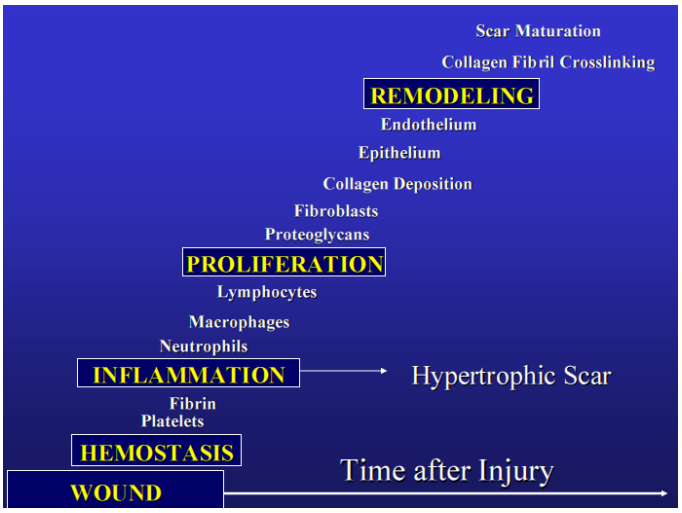

Figure 4. The phases in wound healing with their mediators

Hemostasis Phase

In normal wound healing, hemostasis is a process which stops blood loss via the vascular system to protect the function of the vital organs. This phase consists of bleeding and aggregation. In burn injuries, most of the times, there is no outer bleeding. However, inside the burn wound there are often deep and excessive bleedings. Important mediators in the hemostasis phase are blood vessels, platelets and fibrin.

After injury, vasoconstriction is induced and platelets aggregate and form clots. Due to this clotting process, the complement system is activated. The clot is formed from fibrin, fibronectin, vitronectin and trombospondin, and acts as a matrix for cell migration and proliferation for the cells needed in wound healing. Due to this clot formation, keratinocytes become activated and migrate to the wound matrix. In normal wound healing, fibronectin, a protein which together with fibrin facilitates the migration of fibroblasts, expression decreases after the closing of the wound and endothelial cells induce the destruction of the fibrin clot. *(McDougall et al,2006)(van der Veer, 2008)*

Fibronectin is a glycoprotein which is secreted by fibroblasts and endothelial cells. It has several binding sites for extracellular matrix, including collagen and fibrin, what causes linkages between extracellular matrix. Fibronectin also has a specific cell-binding domain, consisting of three amino acids: Arg-Gly-Asp. This repetition can be recognized by integrin receptors on fibroblasts, and the interaction of these two can cause adhesion, migration and differentiation of fibroblasts to the wound matrix. MMPs assist the migration of fibroblasts and other cells along the fibrils. *(Schultz et al, 2005)* In the wound matrix, fibroblasts are replacing the fibrin of the blood clot with collagen. *(McDougall et al,2006)* Fibronectin also facilitates migration and differentiation of macrophages and neutrophils. *(Schultz et al, 2005)*

In hypertrophic scar tissue, fibronectin deposition is not inhibited after wound closure and fibrin is

not fully removed which leads to an higher fibroblast density and more collagen deposition in the wound matrix. The clot will not be broken down and the attraction of immune cells and fibroblasts is proceeded. *(van der Veer, 2008)*

Inflammation Phase

Both the cellular and humoral immune responses are playing a role in the inflammation stage of wound healing. The immune phase defines the progress of wound healing because in the inflammatory phase an immune barrier is established. At first the complement cascade is activated and neutrophils infiltrate at the site of harm, through margination, rolling and adhesion to endothelial cells. They are stimulated by the mediators released during the hemostasis phase. Neutrophils form the first line of defense and secrete TNF-α and IL-1, which recruit fibroblasts to the wound matrix.*(Schultz et al, 2005)*

After two to three days, macrophages and lymfocytes are attracted by cytokines. These immune cells secrete cytokines, like TGF-β and Platelet Derived Growth Factor (PDGF) which leads to migration of other immune cells, endothelial cells and fibroblasts. The immune reaction leads to vasodilation, cellular permeability and production of extracellular matrix by fibroblasts, which can support migration of several cells. *(van der Veer, 2008)*

In abnormal wound healing, leading to hypertrophic scar formation, there is an overly active inflammation reaction, which leads to an increase in TGF-β and PDGF secretion. This can occur by an increased release of PDGF from platelets, which leads to an enhanced proliferation of fibroblasts and production of collagen. TGF-β can promote fibrosis by catalyzing the transition of several mesenchymal cells (endothelial cells, fibroblasts) into myofibroblasts. When myofibroblasts are activated and they can produce collagen and cause wound contraction.

Macrophages produce several cytokines, including IL-6 and TGF-β. TGF-β induces amongst other things, the release of metalloproteinase enzymes (MMPs) which are able to break down extracellular matrix. In hypertrophic scar formation, the level of TGF-β is increased, which can lead to an accumulation of extracellular matrix, because MMPs are not released. *(Van der Veer, 2008)*

Proliferation Phase

In the proliferation phase, tissue repair is promoted, new blood vessels are formed and a granulation tissue is produced. Myofibroblasts and fibroblasts are attracted to the place of the burn wound by TGF-β and PDGF secreted by immune cells. The migration of fibroblasts is determined by two factors, the concentration gradient of the chemotaxic cytokine and the alignment of the extracellular matrix. Fibroblasts bind to the extracellular matrix and migrate along the collagen fibrils. In the wound matrix, fibroblasts differentiate and deposit extracellular matrix. The deposited extracellular matrix can support cell migration and the transition of fibroblasts into myofibroblasts. *(Schultz et al, 2005)*

The deposition of collagen gives integrity and strength to the surrounding tissues. Myofibroblasts contain much α-smooth muscle actin (α-SMA) and play an important role in wound contraction, wound closure and extracellular matrix production. *(van der Veer, 2008)*

Granulation

After the inflammation phase, tissue repair is induced by the formation of granulation tissue. Macrophages attract fibroblasts and endothelial cells to migrate to the wound. The fibronectin supports the migration of these cells to the wound matrix. The fibrin clot is transformed by fibroblasts into connective tissue, consisting of collagen, glycosaminoglycans and proteoglycans. This

tissue is also called the granulation tissue, and is rich in blood vessels. For the formation of granulation tissue, a balance between the degradation and production of extracellular matrix is required. MMPs, enzymes which can break down extracellular matrix, assist in the formation of granulation tissue and play a role in keeping this balance.
In hypertrophic scar tissue, there is an imbalance in the formation and break down of extracellular matrix. The myofibroblasts density is much higher than in normal burn wound healing, which can be a cause of the accumulation of extracellular matrix. Besides, there is an excessive amount of granulation tissue in hypertrophic scar tissue, which gives the scar the reddish and swollen appearance. *(van der Veer, 2008)*

Re-epithelialization
During the proliferative phase, epithelial cells migrate to the wounded site and form a single cell layer over the wound. This process is called re-epithelialization and can help speed up wound closure. This is an important aspect because if wound closure takes longer than three weeks, the chance of hypertrophic scar formation increases. In normal wound healing, this process starts quickly to restore the skin its integrity. *(Larjava et al, 2000)*

Keratinocytes are laying at the wound edges and are activated by several cytokines and migrate, proliferate and differentiate into cells which can form a cross-talk connection with dermal cells. It forms a protective layer covering the wound. Keratinocytes release IL-1, which can activate fibroblasts, attract endothelial cells, other keratinocytes and lymfocytes. These cells secrete several chemical mediators which induce basal and superbasal keratinocytes. Normally, TGF-β, secreted by fibroblasts, switched the keratinocytes back to an inactivated state, and the inactivated keratinocytes inhibit the collagen production of fibroblasts. When the process of wound healing is complete, myofibroblasts and fibroblasts undergo apoptosis. In hypertrophic scars, keratinocytes have more proliferation, differentiation and the stay activated for a longer period of time. The decreased IL-1 secretion and more PDGF expression, which affect the collagen production of fibroblasts. *(van der Veer, 2008)(Larjava et al, 2000)*

Neovascularization
Due to the inflammation reaction, endothelial cells secrete angiopoietin-2. Together with VEGF, this cytokine stimulates endothelial cells of existing capillaries to create intercellular space, which allows MMPs to degrade the surrounding basal lamina. After endothelial proliferation and migration, a new capillary, running from the original capillary into the wound tissue is formed. After wound healing, the capillaries which were formed regresses to create a level of perfusion that is in balance with tissue homeostasis. *(van der Veer, 2008)*
The role of vascularization in hypertrophic scar tissue is still unknown. However, research showed there are differences in the vascularization in normal burn wound healing and hypertrophic scar formation. There is an higher level of angiogenesis in hypertrophic scar tissue, the formed vessels are more dilated but the oxygen concentration is lower. One of the reasons can be insufficient vascular regression or ongoing angiogenesis, or an combination of these both. An imbalance in the secretion of VEGF and TGF-β can also cause the augmented vascularization of hypertrophic scar tissue. *(Velnar et al, 2009)*

Remodeling Phase

In the remodeling phase there is development of new epithelium and the final formation of scar tissue. The network of collagen formed, the formation of collagen fibrils and the assembly, in this process, determines the function of the extracellular matrix. Collagen fibrils in the skin contain collagen Type I and Type III.

Collagen is produced by (myo)fibroblasts. During tissue remodeling myofibroblasts usually replaces hyaluronan (HA) in the scar tissue by decorin. HA is a large polysaccharide which can bind extracellular matrix molecules like collagen. *(JA Travis, 2001)* Decorin is a proteoglycan which has effects on cell growth, collagen degradation, fibrillogenesis, angiogenesis and extracellular signalling. It can form an interaction with fibronectine, trombspondin and TGF-β. Decorin can bind collagen Type I, and at the same time TGF-β. It binds collagen Type I via the inner region core proteins of decorin, the glycosaminoglycan chain, and TGF-β via the protein core. Patients with a deficiency in decorin have irregular shaped and weaker collagen. *(Ferdous et al, 2007)*

The binding of decorin and TGF-β leads to the regulation of collagen fibrillogenesis and TGF-β by inhibiting the effects of TGF-β. Decorin stimulates the collagen degradation. On the contrary, hypertrophic scar tissue has a lower level of decorin, less TGF-β bound to decorin and more HA in the extracellular matrix. TGF-β is available in unbound form to mediate its function on fibroblasts. *(van der Veer, 2008)*

Collagen Cross-links

The structure of collagen also depends on the types of crosslinks formed between the collagen molecules in the fibril. This differs between tissues, dependent on the function and mechanical load of the tissue.

Recently, there was found that a specific type of not degradable cross-links is present in hypertrophic scar tissue. These cross-links are called pyridinoline cross-links. In normal skin, cross-links are formed due to the oxidation of a lysine residue of the collagen telopeptide into a lysine aldehyde. The lysine aldehyde can react with other lysine residues on collagen telopeptides and in this way they form cross-links. These cross-links are degradable by MMPs. *(van der Veer, 2008)*

However, lysine residues can be transformed in hydroxy-lysine residues. This reaction is catalyzed by lysyl hydroxylase 2 (LH2). The hydroxy-lysine residues are oxidized into hydroxy-lysine aldehydes and pyridinoline cross-links are formed. Pyridinoline cross-links cannot be degraded by MMPs.

Research showed, that TGF-β stimulated (myo)fibroblasts, have a higher level of expression of LH2, and more pyridinoline cross-links are formed. This can be a reason of the increased deposition of collagen in hypertrophic scar tissue.

Another reason for the accumulation of collagen in hypertrophic scar tissue is the imbalance between MMPs, which can breakdown extracellular matrix, and their inhibitor; Tissue Inhibitors of Metalloproteinases-1. (TIMP-1) In hypertrophic scar tissue, the level of MMPs present is decreased, while the expression of TIMPs is increased. This leads to a decrease in degradation of extracellular matrix, due to the inhibition of the MMPs. *(van der Slot, 2004)(Van der Slot, 2005)*

Apoptosis

At the end of each wound healing phase, the cells present disappear by apoptosis, to make space for the cells necessary for the next phase. This also happens in the remodeling phase, after re-epithelialization and wound contraction, myofibroblasts undergo apoptosis. However, in hypertrophic scar tissue, there is still an high density and prevalence of myofibroblasts in the scar tissue. *(van der Veer, 2008)*

Research showed that the Akt-pathway may play an important role in the inhibition of the apoptosis of myofibroblasts. The surface of fibroblasts, TGF-β the cytoskeletal signalling via integrines and the focal adhesion kinases (FAK), activate the Akt-pathway by phosphorylation. This gives an indication that the mechano responsiveness of fibroblasts may induce the inhibition of the apoptosis of myofibroblasts. *(Hinz et al, 2010)*
The Akt-pathway can stimulate cell-survival by acting as a proto-oncogene. It can inhibit apoptosis in several ways.

- Phosphorylation of Bad protein, which inactivate the bcl-2 proto-oncogene. The protein products of this oncogene protect cells from apoptosis.
- Inhibition of the pro-apoptic p53 pathway. A transcription factor which regulates the cell cycle.

The decreased apoptosis of myofibroblasts in hypertrophic scar, leads to an accumulation of fibroblasts at the wound matrix, which in turn leads to an imbalance between the catabolic and anabolic state of extracellular matrix remodeling. The epithelial strength and integrity of the skin is not restored, there is hypercellularity of myofibroblasts and an high collagen deposition. *(van der Veer, 2008)*

The wound healing process is a very dynamic and complex mechanism. Many different defects can cause the abundant deposition of collagen. However, one of the main reasons of the formation of hypertrophic scar tissue is the increased presence of (myo)fibroblasts. The raised density and high prevalence of (myo)fibroblasts can be caused by defects in the different phases and mechanisms of wound healing. Not only does these defects in the mechanisms show us how wound healing in burn injury takes place, it also shows us potential inhibition targets to prevent the formation of hypertrophic scars.

Immune Reponse, Cytokines, Proteases and Scarring

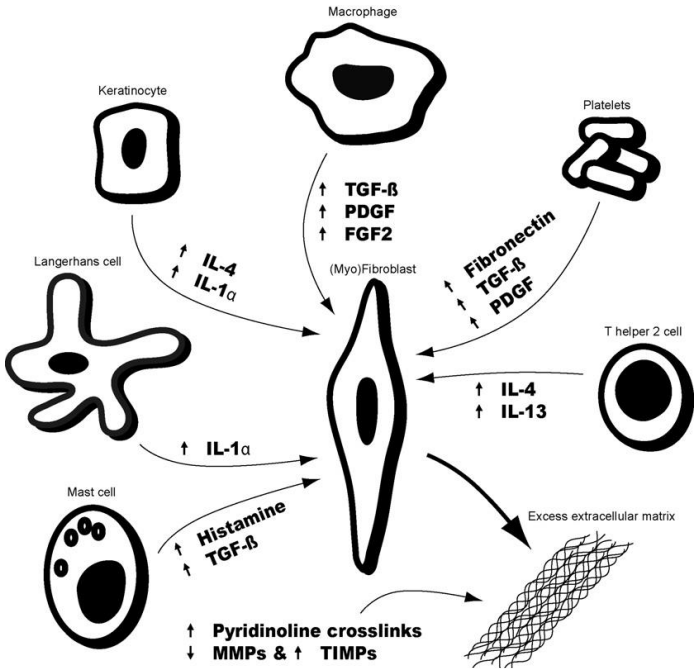

Figure 5. Cells, Cytokines, Growth Factors and other Mediators influencing Fibroblasts during Hypertrophic Scar Formation

During the formation of hypertrophic scar, the immune reaction is enhanced, especially the Th2-lymfocyte immune response, which leads to an increase of the secretion of profibrotic cytokines. Several processes in the wound healing process are mediated by cytokines and growth factors. If the secretion of these cytokines alters, for example the secretion of a cytokine isn't inhibited or another cytokine profile is stimulated, wound healing can fail. Cytokines and growth factors play an important role in the interaction between different cell types. They can attract, inhibit and stimulate other cells or itself. In hypertrophic scar, the influence of the cytokines on fibroblasts is enhanced proliferation, differentiation and deposition of collagen. The collagen being formed determines the architecture of the wound. The formation of pyridinoline cross-links in collagen, results in the stiff and inflexible scar tissue. The balance between MMPs and TIMPs also play an important role in the determination of the extracellular matrix structure. Figure 5 shows the cells, cytokines and other mediators, which influence fibroblasts in hypertrophic scar formation.
Knowing how interactions between cells and the influences of enzymes and proteins in the wound healing process works, will give a better understanding of abnormal wound healing processes and how to treat and prevent them. In the following section, I am going to discuss the Th2-lymfocyte response, a few important pro- and anti-fibrotic cytokines and the role of cross-linking, MMPs and TIMPs in collagen structure.

The Th2 cytokine profile

Hypertrophic scar formation is correlated with an increased and prolonged inflammatory respons. Studies have shown that the formation of hypertrophic scars can be decreased if the wound is healed more rapidly. In the previous section the difference between the normal immune reaction in burn wound healing and the immune reaction of hypertrophic scar formation was stated. During hypertrophic scar formation, the inflammation phase of wound healing is increased, more TGF-β is secreted from immune cells and IL-1 levels decreased.

Burn injury causes a local immune response. The amount of macrophages, platelets, mast-cells, T-cells and Langerhans cells is increased in hypertrophic tissue, while B-cels are not present. Mast cells secrete more IL-4, leading to the differentiation of Th2-lymfocytes. Studies suggested that during hypertrophic scar formation, the profibrotic Th2 CD4+ immune response is exaccerated, while CD8+ T-lymfocytes are present in lower concentrations. *(Gauglitz et al, 2011)(Tredget et al, 2006)*

CD4+ Th2 lymfocyte response

During hypertrophic scarring, antigen presenting cells like Langerhans cellen and fibrocytes are present in higher concentrations, they can activate the CD4+ T-cell in an antigen depending way. Fibrocytes have an overexpression of the costimulating protein B7, which can bind CD28 on the CD4+ T-cell. After activation, the CD4+ T-cell can differentiate into different effector T-lymfocytes with a different cytokine profile: the Th1- and Th2-lymfocytes

IL-12 stimulates naïve CD4+ Th-cells to differentiate intoTh1-lymfocytes, with the Th1 cytokine profile. The Th1-lymfocytes cause an antifibrotic immune response and secrete IFN-ɣ and Interleukin-12. IFN-ɣ is an antifibrotic cytokine and suppresses the deposition of collagen by regulating the balance between matrix metalloproteinases (MMPs) and Tissue Inhibitor of Metalloproteinases (TIMPs). IL-12 reduces fibrosis by downregulating the Th2 cytokine response. However, the IL-10 and IL-4, cytokines of the Th2-lymfocyte response, inhibit IL-12. *(Fibrocytes: New Insight in Tissue Repair and Systemic Fibrosis, 79-81)*

Th2-lymfocytes secrete IL-4, IL-10 and IL-13. IL-4 also stimulates the naïve CD4+ Th-cell to differentiate into a Th2-lymfocyte. The Th2 response is a profibrotic response and it leads to the increase of collagen synthese, deposition, fibroblast proliferation and differentiation and stimulates Th2 lymfocytes to produce more cytokines. The Th2 response and the fibrocytes affect eachother by a positive feedback loop, stimulated by TGF-β. The Th2 response causes a shift in the L-arginine balance between arginase-1 (ARG1) and nitric oxide synthase-2 (NOS2) toward ARG1. The Th2 resonse stimulates the ARG1 response which catalyzes the conversion of L-arginine into L-ornithine, which is the substrate of polyamine and L-proline. Polyamines play a role in cell homeostasis and proliferation. L-proline is an important amino acid for collagen synthesis and is a part of the Gly-Xaa-Yaa repetition in the α-chain of collagen. In this way, the Th2 stimulates collagen synthesis. *(Fibrocytes: New Insight in Tissue Repair and Systemic Fibrosis, 79-81)*

Anti-fibrotic Interleukines and Interferon-ɣ

Interleukins consist of a big family which play a role in leukocyte communication. However, Interleukins can have an influence, besides on the immune response, on the fibrotic state, IL-12 is, for example, an antifibrotic cytokine. IL-12 is produced by keratinocytes and activates immune cells, amongst them macrophages and neutrophils. IL-12 has the following effects: *(Trinchieri, 1995)*

- IL-12 stimulates differentiation of CD4+ T-lymfocytes to the Th1-cell phenotype and cytokine profile.
- IL-12 stimulates natural killer cells.
- IL-12 stimulates CD8+ T-lymfocytes to produce IFN-ɣ, which has many anti-fibrotic and anti-angiogenic downstream effects.
- IL-12 is an chemoattractant for macrophages.

Another anti-fibrotic cytokine is Interferon-ɣ (IFN-ɣ), which is a soluble cytokine which plays a role in the innate and adaptive immune response. It is secreted by CD4+ Th1-lymfocytes. IL-12 has an enhancing effect on wound healing and can decrease fibrosis. It stimulates macrophages to migrate to the wound matrix, it inhibits fibroblast proliferation and collagen production and inhibit the deposition of collagen by regulating the balance between MMPs and TIMPs. At last, IFN-ɣ, can downregulate the Th2-cell profybrotic cytokine response. During hypertrophic scarring, the levels of

IFN-ɣ are decreased.*(Granstein et al, 1990)*
IL-12 and IFN-ɣ are both cytokines secreted during the antifibrotic Th1-lymphocyte response.

Profibrotic Interleukines
The two most important interleukines in the profibrotic Th2-cell response are IL-1 and IL-4They are secreted from keratinocytes, Langerhans cells, neutrophils, and Th2-lymfocytes. IL-1 is the first signal the body receives after dermal burn injury and mediates inflammation by stimulation of clotting. It is first secreted by keratinocytes and has paracrine and autocrine effects. It enhances keratinocyte migration and proliferation and it activates fibroblasts. *(Barrientos et al, 2008)*
IL-4 is secreted by Th2-lymfocytes and mast cells and stimulates the synthesis of extracellular matrix by fibroblasts, this can lead to the abbundant deposition of extracellular matrix, which is a characteristic of hypertrophic scars.*(Salmon-Ehr et al, 2000)*

In normal wound healing, the Th2 response has a reprogramming pathway, which reverses the CD4+ T-cell response and keeps the Th1 and Th2 cytokine response in balance. The reprogramming pathway, stimulates the production of MMPs, which can breakdown abbundant collagen. This is a natural way to prevent fibrotic disorders.
During hypertrophic scarring, the Th2 cytokine response is increased, leading to the proliferation and stimulation of collagen deposition by fibroblasts. The Th1 cytokine profile is inhibited, which normally regulates an anti-fibrotic response.

TGF-β and Scarring

In all the phases of wound healing, Transforming Growth Factor-β (TGF-β) is an important mediator. It plays a role in several mechanisms, including the excessive angiogenesis, the decreases apoptosis of myofibroblasts, the activation of fibroblasts, the synthesis and deposition of collagen and the increased immune reaction during hypertrophic scar formation. It is secreted by many cell types involved in wound healing, among them are platelets, fibroblasts, neutrophils, macrophages and keratinocytes. However, studies showed that if there is a loss of TGF-β, wounds are not healing well. In fibroblasts derived from hypertrophic scar tissue, there was an alteration in TGF-β signalling. This shows, that TGF-β is necessary in the wound healing process, and if their effects are altered, there is deficient wound healing. *(Penn et al, 2012)*

TGF-β is a growth factor and has three isoforms; TGF-β1, -β2 and –β3. The isoforms are located on different genes, however, research showed that they can activate the same intracellular signalling pathway and have overlapping biological functions, and all three play a role in wound healing. Many TGF-β signalling pathways are dependent on the SMAD signalling. SMAD signalling is essential for the induction of several profibrotic effects. But, there are some pathways which are SMAD independent. *(Penn et al, 2012)*

SMAD dependent TGF-β signalling
TGF-β can activate the signalling pathway after binding to the heteromeric TGF-β receptor in the cell membrane. There are two TGF-β receptors, which are both serine-threonine kinase receptors: TβRI and TβRII. After binding of TGF-β on TβRII, TβRI is trans-phosphorylated. The phosphorylation of the receptor complexes recruit several adaptor proteins and phosphorylates SMAD-2 and SMAD-3. The activation of SMAD-2/-3 recruits SMAD-4 and they form a complex. This complex can migrate to the nucleus and bind SMAD binding elements on the DNA. The activation of the SMAD signalling pathway

leads to regulation of several genes, and most importantly, it leads to the elevation of the expression of the gene for extracellular matrix. *(figure 6)* The activation of SMAD dependent TGF-β signalling can be inhibited by the induction of SMAD7 and stimulated by the induction of Connective Tissue Growth Factor (CTGF) *(Leask et al, 2004)*

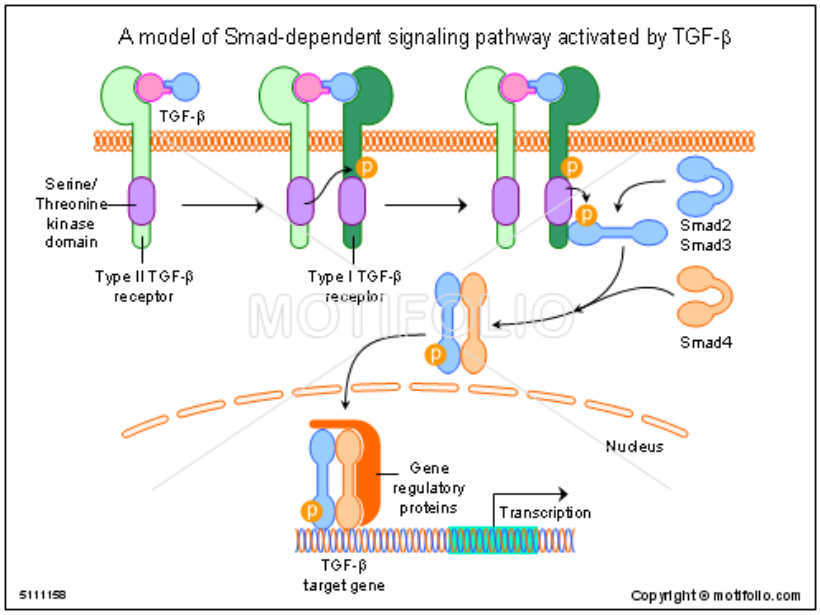

Figure 6. SMAD dependent TGF-β Signalling Pathway

SMAD independent TGF-β signalling

The SMAD independent signalling pathways are essential for the induction of fibronectine and CTGF. The pathway for the induction of fibronectine uses the JNK/MAP kinase and c-Jun signalling pathway to transduce the signal. The RAS/MEK/ERK/MAP kinase pathway is a profibrotic pathway which induces CTGF expression. It does not need SMADs to get activated, but after activation it can modulate SMAD activity for the best transcriptional response. *(Barrientos et al, 2008)(Gui et al, 2012)*

When TGF-β binding leads to the formation of the TGF-β receptor complex, ShcA, an adopter protein, can be phosphorylated. ShcA can then form a complex with Grb and Sos in the cytoplasm. The ShcA complex formation activates RAS, which in his turn can start the ERK/MAP kinase cascade. *(Leask et al, 2004)*The RAS/MEK/ERK/MAP kinase pathway is necessary for the phosphorylation and modification of SMAD activity in the nucleus and can in this way modulate CTGF expression. ERK has a phosphorylation site on SMAD-3 and 4, this phosphorylation is necessary to obtain the maximal transcriptional response. *(Chen et al, 2002)(Moustakas et al, 2005)(Kolch et al, 2000)*

TGF-β in Hypertrophic Scars

In hypertrophic scar tissue, it has shown that fibroblasts have an higher and prolonged TGF-β expression. In normal wound healing, the TGF-β receptor expression decreases in the remodeling phase. The increased expression of TGF-β probably leads to a positive feedback loop. TGF-β induces fibroblasts proliferation, differentiation and collagen deposition, which can produce TGF-β. Finally results in stimulating the fibrotic phenotype. Patients, forming hypertrophic scars have a high plasma level of CD4+ TGF-β producing T-cells. Besides increased TGF-β in fibroblast from hypertrophic scar tissue, there is also an increased expression and phosphorylation of the SMAD-2 and SMAD-3 proteins. Which play a role in the signalling pathway of TGF-β. *(Penn et al, 2012)* Besides, TGF-β controls the expression of several integrins, and in fibrotic disorders the expression of certain integrines which can mediate collagen remodeling and myofibroblast contraction increases. *(Margadant et al,2010)*

In recent studies was shown that proteoglycans also have an interaction with TGF-β. Proteoglycans, such as decorin, play a role in cell signalling and interaction and modulation of extracellular matrix. In hypertrophic scar tissue, the decorin expression is decreased. Decorin can bind all the three isoforms of TGF-β, and they inhibit the actions of TGF-β due to this binding to extracellular matrix. In hypertrophic scar tissue, the level of decorin proteoglycan is decreased, which can lead to an abnormal collagen organization and an increased production of extracellular matrix compounds. If decorin is decreased, TGF-β cannot be inhibited, and will induce fibroblasts to produce and deposit collagen. *(Penn et al, 2012) (Ferdous et al, 2007)*

TGF-β Downstream Mediator Connective Tissue Growth Factor
Connective Tissue Growth Factor (CTGF) is an cysteine-rich heparin-binding peptide secreted by fibroblasts. In normal conditions, CTGF is not present in the skin. During the wound healing process, CTGF is induced and the levels are elevated. CTGF can be induced in fibroblasts by TGF-β, but does not occur in keratinocytes. It has a function in embryogenesis, wound healing and the regulation of extra cellular matrix production, and is seen to be overexpressed in several fibrotic disorders. *(Oemar et al, 1997)* CTGF influences fibroblasts by inducing cell-cell adhesion, migration, chemotaxis, proliferation, the production of the extra cellular matrix components collagen and fibronectin, the contraction of collagen matrix and the formation of granulation tissue. CTGF also induced the re-epithelialization, the remodeling phase of wound healing and angiogenesis by stimulating endothelial migration. This shows that CTGF plays an important role in the wound healing process. *(Leask et al, 2004)*

In the promoter of the CTGF gene, a TGF-β response element was found, which shows CTGF can be induced by TGF-β. After the stimulation of TGF-β, CTGF mediates many of the effects on fibroblasts. Researchers looked at wound repair in wound matrixes which were injected with TGF-β and CTGF. In both conditions, a fibrotic response took place, which shows us that TGF-β and CTGF both have fibroblast-activating characteristics and that CTGF acts as a downstream mediator of TGF-β in fibrosis. Studies using neutralizing antibodies which can bind CTGF and recombinant CTGF showed that CTGF has indeed downstream effects on fibroblasts. *(Denton et al, 2001)*
In the formation of hypertrophic scar tissue, the level of CTGF in fibroblasts is about 20 times higher than in normal fibroblasts. *(Barrientos et al, 2008)* Researchers showed, that the inhibition of CTGF, decreased the formation of hypertrophic scars but did not interfere with wound closure. *(Penn et al, 2012)* This makes CTGF a possible inducer of collagen synthesis in fibroblasts. *(Duncan et al, 1999)*

Proteases and the Extracellular Matrix

There are four types of proteolytic enzymes which can breakdown extracellular matrix. These are matrix metalloproteinases (MMPs), serine proteinases, cysteine proteinases en aspartic proteinases. MMPs are needed in the degradation and remodeling of collagen. MMPs and their inhibitor, Tissue Inhibitors of Metalloproteinases (TIMPs), play an important role in the regulation of the collagen remodeling in the wound healing process. MMPs can break down collagen on specific regions of the fiber and can change the orientation and structure of collagen in this way. The catalytic domain of MMPs can unwind the triple helixes of collagen and can cleave the strands one by one. In the human body, 23 MMPs are known. MMP -1, -8, and -13 can hydrolyze the collagen after a glycine-residue in the collagen chain, which is at ¾ of the N-terminal. This leads to two specific fragments. These fragments can be further degraded by MMP-2 and -9. *(van Marion, 2006)*

MMPs are regulated by TGF-β, TNF-α and Interleukins. These cytokines and growth factors regulate the gene expression of MMPs. However, MMPs can also be regulated by the mechanical stretch in the skin. Collagen has many mechanical contributions to the skin, this depends on the structure of the collagen fibrils. The cross-links between the fibrils stabilizes them, and makes the collagen stronger. So, if the balance between MMPs and TIMPs is destroyed, it will affect not only the content of the collagen but also the mechanical properties of the skin. *(van Marion, 2006)*
In hypertrophic scar tissue the balance between degradation of collagen and the inhibition of degradation is disturbed. This imbalance can cause the excessive accumulation of extracellular matrix. *(Widgerow, 2013) (Qiu et al, 2003)* Normally, collagen can be broken down by MMPs. MMPs can be inhibited in three ways:

1. Inhibiting the protein expression and synthesis of MMPs
2. Inhibition of the mechanism which activates MMPs
3. Or the inhibition of the active MMPs.

Active MMPs can be inhibited by binding TIMPs. TIMPs can bind their active site and neutralize the downstream effects of MMPs. Patients with hypertrophic burn scars, have an higher expression of TIMPs. This means, more MMPs are inhibited, and the collagen in the hypertrophic scar tissue cannot be degraded. The overexpression of TIMPs can lead to fibrotic symptoms of the hypertrophic scars, by inhibiting the active MMP. *(van Marion, 2006) (Widgerow, 2013)(Qiu et al, 2003)*
However, not only the elevated level of TIMPs is the problem in hypertrophic burn scars, there is also the formation of collagen cross-links which cannot be broken down by MMPs. These cross-links are called pyridinoline cross-links.
Pyridinoline cross-links are usually found in bone and cartilage tissue, and does not occur normally in the skin. However, studies showed, pyridinoline cross-links also occur in hypertrophic scar tissue. The formation of pyridinoline cross-links can cause the accumulation of extracellular matrix, because they cannot be degraded by MMPs.

There are two pathways for cross-link formation *(figure 7)* the allysine and hydroxyallysine pathway: In the allysine route, a lysine residue in the collagen telopeptide is oxidized by lysyl oxidase (LOX) into an lysine aldehyde. The lysine aldehyde can form cross-links with lysine-residues or hydroxy-lysine residues on other collagen telopeptides. In the hydroxyallysine route, an hydroxy-lysine residue in the collagen telopeptide is oxidized by LOX into an hydroxy-lysine aldehyde, which can also form cross-links

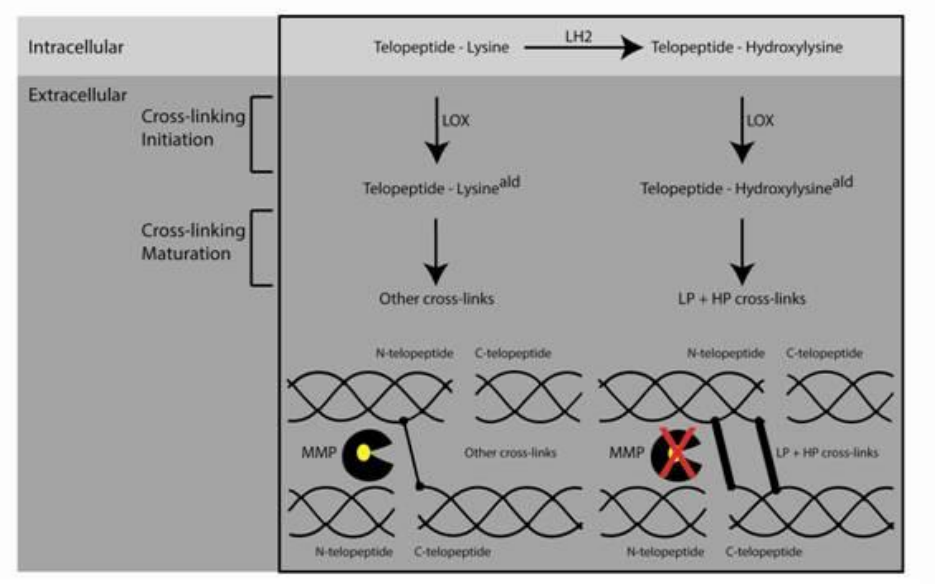

Figure 7. The Allysine and Hydroxyallysine Cross-linking Pathway in Collagen, M. Boersema

with other lysine- and hydroxy-lysine residues. However, the cross-links formed from this route, cannot be degraded by MMPs.

During fibrotic disorders, there is a switch from the allysine route to the hydroxyallysine route, increasing the formation of pyridinoline cross-links. This switch can occur due to an over hydroxylation of lysine residues. *(van der Slot, 2004)(Van der Slot, 2005)* Lysine residues in the telopeptides, can be converted into hydroxylysine residues. This reaction is catalyzed by lysyl hydroxylase 2 (LH2). Research showed that in hypertrophic scar tissue, there is an increased expression of LH2, leading to an enhance formation of pyridinoline cross-links. *(van der Veer, 2008)* Inhibition of formation of pyridinoline cross-links, can decrease the accumulation of extracellular matrix and the formation of hypertrophic scar tissue.

Restoration of Hypertrophic Scar Tissue

The biggest problems in hypertrophic scarring is the hypercellularity and the excessive deposition of extracellular matrix. In the previous sections, I discussed a few mechanisms which fail in hypertrophic scarring. In short, the wound healing phases are altered and affected, there is an increased Th2-lymfocyte inflammation response, excessive extracellular matrix deposition, increased angiogenesis, longer phase of re-epithelialization, a decrease in degradation of extracellular matrix and a reduced apoptosis of (myo)fibroblasts. These are all potential targets for the treatment or prevention of hypertrophic scars.

Every deep dermal wound will heal with a scar, however when the normal wound healing process has finished, the scar is close, is not inflammated and the skin is flat. Hypertrophic scars are raising above the skin, is red and inflammated and gives physical and psychological harm to the victim. The only process in which there is still scarless wound healing, is in the fetal wound healing process.

Fetal Wound Healing

In comparison to adult wound healing, fetal wounds heal scarless and very fast. This shows us that scar formation is age dependent. There are many differences between the wound healing process in fetal tissue and in the adult skin.
First of all, in fetal wound healing there is a lower level of inflammation and a lower level of immune cells present. Thereby, the time the cells are presence is short and they are less activated. Besides all the expression levels of the mediators, cytokines and growth factors are decreased in the fetus. The collagen synthesis differs from the adult in speed, the mechanism and the structure of collagen. In fetal tissue, the collagen is deposited in a fine woven pattern, while in adult wound healing it is less organized. Also the level of HA in the extracellular matrix is much higher.
The levels of urokinase plasminogen activator and MMPs are increased, and the level of their inhibitors are decreased. The TGF-β expression is lower in fetal wound healing and TGF-β is cleared very fast. *(Rolfe et al, 2012)*

During fetal wound healing, regeneration is promoted instead of repair. The regeneration leads to the scarless wound healing. Compared to fetal wound healing, all the mechanisms of the wound healing process is adults are overly active and delayed. The fetal wound healing process is more delicate and faster. *(Rolfe et al, 2012)*

Wound healing leading to an hypertrophic scar is a very complex and dynamic mechanism, with many factors which can be altered, which can be used as intervention but still no treatment available. The excessive collagen accumulation may have several reasons and will not be solved by one intervention. The biggest problem in finding a treatment for the healing of burn wounds is that there is no good animal model of hypertrophic scar tissue available. Human trials are necessary for the best results. Thereby, there are many differences in the wound healing mechanism between individuals. *(Gauglitz et al, 2011)* In my opinion, the best way to get the ideal wound healing result, is to work towards an wound healing process which is much like the fetal wound healing state, promoting regeneration instead of repair. However, much research have to be done and every little step toward repair of the hypertrophic scar by intervening single cell mechanisms in the wound healing process will help burn injury victims to defeat their injury. Full regeneration will hopefully be a future prospective.

Opportunities for Intervention

In the wound healing process burn injury, specialists understand the pathophysiology, which processes and mechanisms are wrongly regulated and but still there is no intervention method available. Victims from burn injury still encounter several dysfunctions and disfigurements due to hypertrophic scars. Current treatments can reduce the inflammation and the pain, but the scar still exists. The ideal result of the treatment of hypertrophic scarring is regeneration of the skin to a pre-injury state. However, this is a distant result, inhibition of the formation of hypertrophic scars is a more realistic objective. This will probably cause less psychological and physical problems for the victims. To heal a burn wound without hypertrophic scars, the alternate wound healing process to hypertrophic scar formation has to be changed back to normal wound healing process. The first step is to change single cell mechanisms back to their normal state.

Induction of the Th1-lymfocyte response

In hypertrophic scar formation, the Th2 cytokine profile is stimulated. The Th2 cytokines inhibit IL-12, so naïve CD4+ Th-cells cannot differentiate into Th1 cells. CD4+ T-helper cells are stimulated to differentiate into Th2-lymfocytes. The Th2-lymfocytes have an pro-fibrotic cytokine profile, inducing fibrosis and is elevated during hypertrophic scar formation. In normal wound healing, there is a balance between the Th1- and Th2-lymfocyte response. The Th1-lymfocytes have an anti-fibrotic cytokine profile, secreting IL-12 and IFN-ɣ.

Normally, the CD4+ Th-lymfocytes are stimulated by IL-12 to differentiate into CD4+ Th1-lymfocytes, which in turn can produce and secrete IFN-ɣ. IFN-ɣ stimulates more naïve CD4+ T-cell differentiation into Th1-lymfocytes and it blocks the development of Th2-lymfocytes. *(Herndon, Total Burn Care)*

To induce the Th1-lymfocyte response, the levels of IL-12 and IFN-ɣ have to be increased. This will lead to differentiation to the Th1 cytokine profile, inhibition of the Th2-lymfocyte response, and secretion of anti-fibrotic and anti-inflammatory cytokines. By stimulation of IL-12 producing macrophages, the IL-12 levels can rise and induce secretion of IFN-ɣ. *(Bucala)*This may lead to inhibition of hypertrophic scar formation by suppressing the collagen deposition and inhibiting the effect of pro-fibrotic cytokines.

Stimulation of MMPs

Matrix Metalloproteinases degrade collagen. In hypertrophic scarring, MMPs are inhibited in several ways. The level of IL-1, a cytokine which induces the release of MMPs, is decreased, and more TIMPs, MMP inhibitors, are present in hypertrophic scar tissue. MMPs can prevent the accumulation of extracellular matrix in hypertrophic scarring, which prevents the raised scar formation.

MMPs can be induced by stimulation of MMP synthesis, MMP activation, raising the levels of IL-1 or inhibition of TIMPs. For hypertrophic scarring, I think, the inhibition of TIMPs is the most effective solution. In hypertrophic scar tissue, there is an imbalance between TIMPs and MMPs, MMPs are present in the scar tissue but the high level of TIMPs inhibit their effect. Using neutralizing anti-TIMP antibodies, which can bind TIMPs making them unable to bind the active site of MMPs, the effect of TIMPs will be diminished and MMPs are able to break down collagen. *(van Marion, 2006) (Widgerow, 2013)(Qiu et al, 2003)* However, inhibition of TIMPs can cause little inhibition of the degradation of collagen, so not all the TIMPs must be inhibited. You can temporarily inhibit the activity of TIMPs by

the use of protease inhibitors which bind weakly to the active site of TIMPs and can be easily replaced to activate them again.

Inhibition of TGF-β secretion
TGF-β is the most important fibrotic mediator of hypertrophic scarring. It has several effects in the wound healing process leading to hypertrophic scars, amongst them synthesis and deposition of collagen, activation of fibroblasts and the promotion of angiogenesis. TGF-β is secreted by several different types of cells. CTGF, a downstream mediator of TGF-β, is also an pro-fibrotic cytokine.

TGF-β is a complex target for the prevention of hypertrophic scarring, because excessive presence of TGF-β leads to an accumulation of extracellular matrix, however, a decrease of TGF-β leads to non-healing wounds. This shows, TGF-β is needed in the wound healing process, and inhibition of the entire effects will also lead to deficient wound healing. Neutralizing antibodies will block both the fibrotic and immune response of TGF-β.
The goal of TGF-β inhibition is just to block the fibrotic response of TGF-β, and not the effects of TGF-β on the immune response. The TGF-β fibrotic response can be modulated by increasing the presence of natural inhibitor of TGF-β. For instance, decorin, which can bind the active site of TGF-β so it is not able to mediate its downstream effects. Another way to block TGF-β signalling, is to block the TGF-β signalling pathway. The use of an TGF-β receptor antagonist, will block the fibrotic effects of TGF-β on fibroblasts. Also SMAD signalling and Ras/Raf/MEK/ERK signalling can be targets to be inhibited to modulate TGF-β effects, this can be mediated with a protein kinase inhibitor. *(Penn, 2012)*

Prevention irreversible cross-link formation
Research showed, hypertrophic scar tissue had an increase of pyridinoline cross-links in the extracellular matrix. Pyridinoline cross-links are formed, when a lysine residue in the telopeptide of collagen is hydrolyzed into an hydroxy-lysine residue. This hydroxy-lysine residue can be oxidized into and hydroxy-lysine aldehyde which can form pyridinoline cross-links with other lysine or hydroxy-lysine residues. The pyridinoline cross-links are not easily degraded by MMPs, leading to accumulation of collagen.
The hydroxylation of the lysine residue to the hydroxy-lysine residue is catalyzed by the enzyme Lysyl Hydroxylase (LH). Normally, degradable cross-links are formed from oxidation of lysine residues. By inhibiting the hydroxylation of lysine residues, degradable cross-links are formed and there will be no accumulation of extracellular matrix. This conversion can be inhibited by blocking the hydroxylation by LH2.

LH2 can be inhibited by decreasing the mRNA expression, use neutralizing antibodies, or by depletion of the required cofactors vitamin C and iron. mRNA expression can be decreased by blocking the translation of the LH2 gene. Neutralizing antibodies can bind the active site of LH2 and inhibit the hydroxylation of lysine residues. LH2 need vitamin C and iron as cofactors for the hydroxylation, if these cofactors are not available or cannot bind LH2, LH2 cannot hydrolyze lysine residues. *(van der Slot, 2004)(Van der Slot, 2005)*

Stimulation of Myofibroblast Apoptosis. In normal wound healing, after the wound closure, (myo)fibroblasts undergo apoptosis. However, in hypertrophic scarring, apoptosis of (myo)fibroblasts is inhibited and there is an hypercellularity of (myo)fibroblasts with an high prevalence in the harmed tissue. This can be one of the causes of the accumulation of extracellular matrix. Stimulation of

apoptosis, can decrease the level of fibroblasts present in the scar tissue and decrease the collagen deposition.

In hypertrophic scar tissue the Akt pathway is stimulated by TGF-β, leading to the promotion of cell survival and the inhibition of apoptosis. Protein kinase inhibitors can inhibit the Akt signalling pathway in myofibroblasts and induce apoptosis. Protein kinase inhibitors block the phosphorylating activity of the Akt pathway, so kinases cannot be cross-phosphorylated and signals cannot be transduced. Protein kinase inhibitors are often used in the treatment of cancer, where hypercellularity is also a big problem, to decrease inflammation, suppress cell growth and induce apoptosis by inhibiting the Akt signalling pathway. *(Yang et al, 2004)*

Transition of Myofibroblast to Fibroblast
In the process of hypertrophic scar formation, fibroblasts are converted into myofibroblasts by TGF-β and mechanical stimuli. TGF-β is seen as a promoter of the myofibroblast phenotype and it triggers the transition. In hypertrophic scars, this transition may lead to excessive extracellular matrix accumulation, contractures and an high prevalence of myofibroblasts.
Inhibition of this transition, can lead to less myofibroblasts in the wound matrix and less collagen deposition. The transition can be inhibited by blocking the effects of TGF-β on fibroblasts and reducing the mechanical signalling by relaxins. IL-1 and IFN-ɣ can inhibit the α-SMA expression induced by TGF-β, and inhibit the fibroblast-myofibroblast transition.
However, another option can be the transition back to fibroblasts. The trigger for this transition is not yet known, however could have great potentials for an target to inhibit hypertrophic scar formation. In hypertrophic scars, the amount of myofibroblast present is increased which causes wound contracture and the abundant deposit of collagen. A cytokine or protein that triggers the transition from myofibroblasts to fibroblasts, will decrease wound contractures occurring in hypertrophic scars. For future research, the transition from myofibroblast-to-fibroblast can be a potential target, questioning if there is a mediator of the fibroblast phenotype and is the transition to the myofibroblast phenotype reversible?

The six ways to intervene or change a mechanism in the wound healing process during hypertrophic scar formation mentioned above, is a way to alter the wound healing process and turn it back to the normal wound healing process. These interventions have to happen early in the formation of hypertrophic scar tissue, and are focused on modulating single cell type effects back to their normal working. These interventions are trying to turn the failed wound healing process leading to hypertrophic scars, step to step, back to the normal wound healing process to decrease the consequences of hypertrophic scars.

Conclusion

Deep dermal burn wounds always lead to scar tissue. When the wound healing process delays, hypertrophic scars can be formed. A Hypertrophic scar is characterized by hypercellularity, an excessive collagen deposition, less elastic extracellular matrix and results in a reddish scar raised above the skin, between the boundaries of the initial burn injury. Hypertrophic scars can cause limitations in mobility, psychological harm due to the prominent scars and dysfunction and disfigurement of the skin. *(Meenakshi et al, 2005)*

The most ideal result of the treatment of hypertrophic scars, is not repairing the burned skin, but regenerating a new scarless skin. Scarless wound healing only occurs in fetal tissue. Compared to normal wound healing, fetal wound healing is much faster, without inflammation and a decreased presence of cells, cytokines and growth factors. *(Rolfe et al, 2012)*This will be a prospective for the future. Modulating the wound healing of hypertrophic scar formation towards the fetal wound healing process is a chance to regenerate the skin of burn victims to the pre-injury state, without the formation of a scar. However, improving burn wound healing with little chance on the formation of hypertrophic scars, will be the first improvement in the future of inhibition of hypertrophic scar formation. Every little step towards inhibiting hypertrophic scar formation in burn injury, will prevent burn victims for a lot of post-injury harm.

To find a treatment against the formation of hypertrophic scar tissue, knowledge of the pathophysiology and how the process differs from normal wound healing is important. The wound healing process can fail and be delayed in several ways, the inflammation reaction can be over active, the apoptosis of myofibroblasts can be inhibited, an altered hemostasis phase, and a changed remodeling phase. All of these changes lead to an abundant deposition of extracellular matrix by fibroblasts. *(van der Veer, 2008)* In these processes, there are many cells, phases or cytokines which can be inhibited or activated, to return the wound healing process to normal.

The main problems in finding a treatment to inhibit hypertrophic scar formation is the lack of a useful animal model and the differences between each individual. The wound healing process differs per individual and is age, race, sex location and type of wound dependent. Which causes problems in finding reliable data because often patient models are used, in which the hypertrophic scar formed months before the trial. However, even small improvements in minimizing scarring can be very meaningful for clinical use. *(Gauglitz et al, 2011)*

Possible treatment targets for the inhibition of hypertrophic scar formation is decreasing the TGF-β levels in the scar tissue, promotion of the Th1 cytokine profile during the immune reaction, stimulating apoptosis of myofibroblasts after wound closure, prevention of the formation of non-degradable cross-links, stimulation of MMPs and transition the myofibroblasts back to fibroblasts. By altering these processes, still a scar will be formed, however, this scar will not be infected, will not be raised above skin level and will probably fade and resolve faster. Only the wound healing will decrease the chance of hypertrophic scar formation, and will have the normal and balanced wound healing phenotype.

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