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Keloids - A fibroproliferative disease

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ABSTRACT

Keloids are a fibroproliferative disorder of unknown etiology developing in the skin after injury or spontaneously. The aim of this thesis is to gain deeper insight into the role of TGF-β and its signaling pathway proteins, SMADs, in the pathogenesis of keloids and describe the gene expression profile in different keloid sites in the search for potential target genes for future treatment. Further aim is to develop an instrument to describe the quality of life of patients with keloids.

We find cultured keloid fibroblasts to express an increased level of TGF-β1 mRNA and a decreased level of TGF-β3 mRNA compared to control skin. Keloid derived fibroblasts exhibit significantly decreased mRNA levels of TGF-β receptor type II (TβRII) and the ratio of TβRI/TβRII mRNA expression is increased. This suggests that a certain expression pattern of TGF-β subtypes and receptors may be important in keloid pathogenesis.

Analysis of keloid derived fibroblasts reveal decreased SMAD3 mRNA expression and decreased ratio of SMAD2/SMAD3 mRNA implicating a disturbed SMAD signaling. Keloid fibroblasts up-regulate SMAD4 protein after stimulation with TGF-β1 and display diminished levels of the inhibitory proteins SMAD6 and 7. This may contribute to unlimited and deregulated TGF-β signaling leading to increased extracellular matrix production (ECM).

The gene expression pattern is described in fibroblasts from different keloid sites using microarrays covering the whole human genome. This study reveals 105 regulated genes (79 genes are up- and 26 down-regulated) resulting in a unique gene expression profile in different sites of keloids, where progression or regression of the keloid process took place. In cells from the central part of keloids with clinical signs of regression, an up-regulation of apoptosis inducing genes as ADAM12 and ECM degrading genes as MMP19 is found. These genes may contribute to regression of keloids and might be possible future target genes for treatment. Overexpression of apoptosis inhibitors as AVEN and down-regulation of angiogenesis inhibiting genes as PTX3 found at the active margin of keloids may be responsible for the invasive character of the keloid margin.

We develop a disease specific questionnaire to measure the quality of life of patients with keloids. We find two scales, psychological and physical impairment, describing the dimensions of quality of life in patients with scars. These two scales are independent of each other and show a high test-retest reliability. Single items which clinically characterize the disease show correlations to these scales. The results of this study demonstrate for the first time a severe impairment of quality of life of patients suffering from keloids and hypertrophic scars.

In conclusion the described alteration in TGF-β expression and its receptors, the disrupted SMAD signaling pathway and the unique gene expression patterns in different keloid sites provide new knowledge on ECM formation and degradation in keloids. Regulatory genes in ECM homeostasis may be future target genes for keloid prevention, regression and treatment. The disease specific quality of life instrument of patients with keloids and scars is a useful tool to estimate success in future therapeutic efforts over time.

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ORIGINAL PUBLICATIONS

This thesis is based on the following original papers, which will be referred to in the text by their Roman numerals (I-IV).

I. **Bock O**, Yu H, Zitron S, Bayat A, Ferguson MW, Mrowietz U
Studies of transforming growth factors beta 1-3 and their receptors I and II in fibroblast of keloids and hypertrophic scars.
Acta Derm Venereol 85:216-20, 2005

II. Yu H, **Bock O**, Bayat A, Ferguson MW, Mrowietz U
Decreased expression of inhibitory SMAD6 and SMAD7 in keloid scarring.
J Plast Reconstr Aesthet Surg 59(1):221-9, 2006

III. **Bock O**, Schmid-Ott G, Malewski P, Mrowietz U
Quality of life of patients with keloid and hypertrophic scarring.
Arch Dermatol Res 297(10):433-8, 2006

IV. **Seifert O***, Bayat A, Geffers R, Buer J, Löfgren S, Matussek A
Identification of unique gene expression patterns within different lesional sites of keloids.
*Oliver Bock has changed surname to Seifert
Accepted: Wound Repair and Regeneration

ABBREVIATIONS

BMP	bone morphogenetic protein
bFGF	basic fibroblast growth factor
CTGF	connective tissue growth factor
ECM	extracellular matrix
EGF	epidermal growth factor
ELISA	enzyme-linked immunosorbent assay
GAPDH	glyceraldehyde-3-phosphate dehydrogenase
HIF-1alpha	hypoxia-inducible factor 1, alpha
HRP	horseradish peroxidase
ICAM	intercellular adhesion molecules
IGF	insulin-like growth factor
IFN-γ	interferon-gamma
IL-1	interleukin-1
KF	keloid fibroblasts
MMP	matrix metalloproteinase
PAI-1	plasminogen activator inhibitor-1
PAF	platelet-activating factor
PBS	phosphate buffered saline
PDGF	platelet-derived growth factor
PMN	polymorphonuclear neutrophils
POMC	propiomelanocortin
RT-PCR	reverse transcription-polymerase chain reaction
TGF-β	transforming growth factor-beta
TβRI, II, III	transforming growth factor-beta receptor I, II, III
TIMP	tissue inhibitor of metalloproteinase
TNF-α	tumour necrosis factor-alpha
t-PA	tissue-plasminogen activator
u-PA	urokinase-plasminogen activator
VEGF	vascular endothelial growth factor

INTRODUCTION

THE SKIN

The integument, or skin, covers the entire external surface of the human body and is the principle site of interaction with the surrounding world. It serves as a protective barrier preventing internal tissues from exposure to trauma, ultraviolet radiation, temperature extremes, toxins, and bacteria. Other important functions of the skin include sensory perception, immunologic surveillance, thermoregulation, and control of insensible fluid loss. The skin is not only the largest organ in the body, but also the heaviest: it has a surface of 1.5–2m^2 and contributes 1/7 to 1/6 of bodyweight. The integument consists of two dependent layers, the epidermis and dermis, which rest on a fatty subcutaneous layer, the panniculus adiposus (Figure 1). The epidermis, the outermost layer, consists mainly of keratinocytes which regenerate at the basement membrane and migrate to the surface. This migration takes about four weeks and results in a corneocyte (horny) layer serving as a protective barrier. Further, the keratinocytes keratinize to produce nails and hairs. Melanins, which are responsible for skin pigmentation, are found throughout the epidermis. The main roles of the dermis, the second layer of skin, are to regulate temperature and to supply the epidermis with nutrient-saturated blood. The main cell population in the dermis are fibroblasts, which produce connective tissue lending elasticity and support to the skin. It is the seat of hair follicles, nerve endings, and pressure receptors. The deepest layer is the subcutaneous tissue containing adipocytes, which provides mechanical and thermal protection and serves as energy storage.

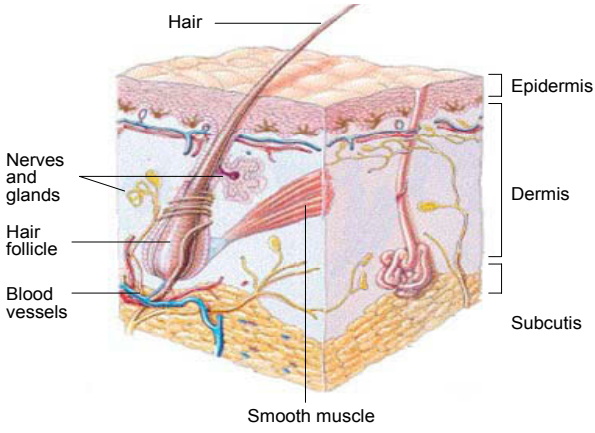

Figure 1. Anatomy of the skin. Modified from Starr & Taggart (1995).

WOUND HEALING

Wound healing is a natural restorative response to tissue injury and requires the interaction of a complex cascade of cellular and humoral events to re-establish the injured skin. Healing is a systematic process explained in terms of three classic phases: inflammation, proliferation, and maturation (Figure 2). During the inflammatory phase a clot forms and inflammatory cells debride injured tissue. Epithelialization, fibroplasia, and angiogenesis occur during the proliferative phase. Meanwhile, granulation tissue forms and the wound begins to contract. Finally, during the maturation phase, collagen develops tight cross-links to other collagen and with protein molecules, increasing the tensile strength of the scar (1).

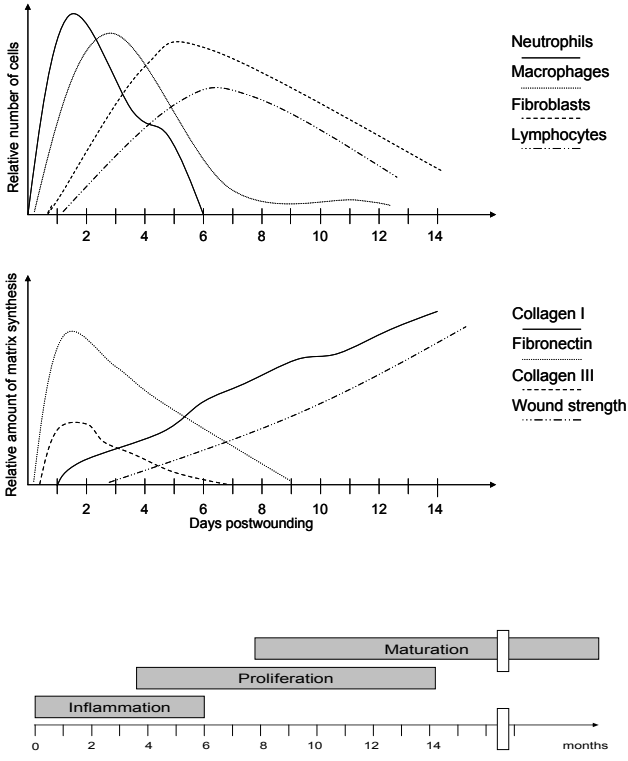

Figure 2. Principles of the cellular and biochemical response in wound healing as described by Brunicardi (Schwart´s Principles of Surgery, 8th Edition, 2006).

Inflammation

The inflammatory phase of wound healing (Figure 3) is preceded by haemostasis. Wounding of the skin disrupts tissue and blood vessels leading to a direct exposure of extracellular matrix to platelets. This is followed by platelet aggregation, degranulation, and activation of the coagulation cascade. Platelets release a number of wound-active substances, such as platelet-derived growth factor (PDGF), transforming growth factor-beta (TGF-β), platelet-activating factor (PAF), fibronectin, and serotonin. The fibrin clot serves as scaffolding for the migration of inflammatory cells into the wound such as polymorphonuclear neutrophils (PMN) and monocytes.

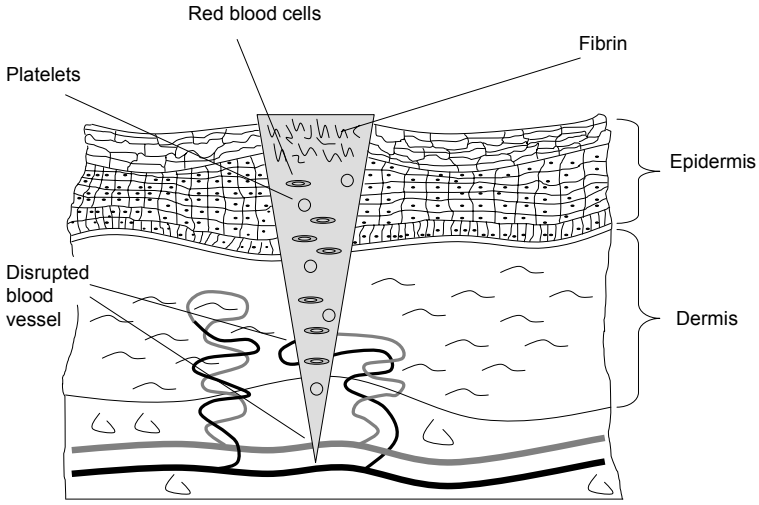

Figure 3. Inflammatory phase of wound healing.

The role of PMNs is phagocytosis of bacteria and tissue debris. PMNs are also a major source of cytokines, especially tumour necrosis factor-alpha (TNF-α), and release proteases such as collagenases, which participate in matrix degradation. The second population of inflammatory cells that invades the wound consists of macrophages. Macrophages participate in wound débridement via phagocytosis and contribute to microbial stasis. The most important function of macrophages is activation and recruitment of other cells via cytokines, growth factors and by cell-cell interaction and intercellular adhesion molecules (ICAM). By releasing mediators as TGF-β, vascular endothelial growth factor (VEGF), insulin-like growth factor (IGF) and epidermal growth

factor (EGF), macrophages regulate matrix synthesis, cell proliferation and angiogenesis (2). The third cell population invading the wound are T-lymphocytes representing the transition from the inflammatory to the proliferative phase of healing. The role of lymphocytes in wound healing is not fully understood (3). Depletion of T-lymphocytes decreases wound strength and collagen content (4). Lymphocytes exert a downregulating effect on fibroblast collagen synthesis by the production of interferon-gamma (IFN-γ), TNF-α and interleukin-1 (IL-1) (5).

Proliferation

The second phase of wound healing is the proliferative phase spanning from 4 to 12 days after injury (Figure 4). During this phase tissue continuity is re-established. The last cell populations infiltrating the wound are fibroblasts and endothelial cells. PDGF is the strongest chemotactic factor for fibroblasts (6). The primary function of fibroblasts is matrix synthesis and remodeling. As part of the angiogenetic process endothelial cells proliferate extensively during the proliferative phase of wound healing and migrate into the wound from adjacent venules stimulated by cytokines and growth factors (TNF-α, TGF-β and VEGF) (7).

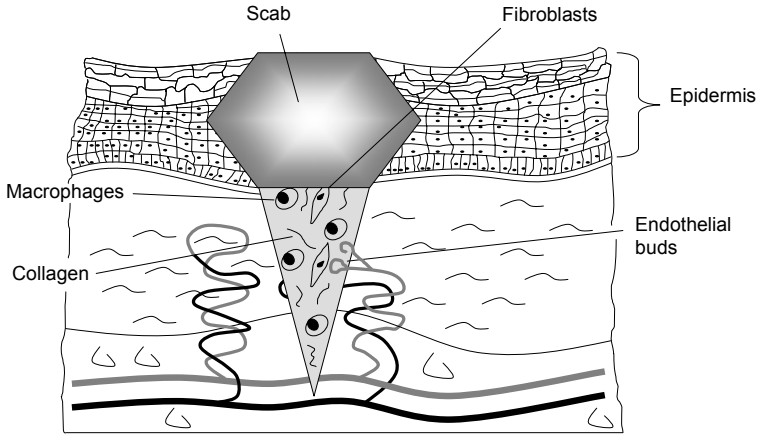

Figure 4. Proliferative phase of wound healing.

The early granulation tissue develops in the proliferative phase of wound healing and is characterized by the presence of myofibroblasts. They contain an elevated level of α-smooth muscle actin and are morphologically and functionally intermediates between fibroblasts and smooth muscle cells (8). Myofibroblasts are attributed to wound tightening and their persistence may lead to abnormal scar contraction.

Maturation

The maturation phase and remodeling of the scar is characterized by a reorganization of previously synthesized collagen. The wound collagen content is a result of the balance between collagen synthesis and collagenolysis. The quantity and quality of the newly produced collagen determines wound strength and mechanical integrity. Fibronectin and collagen type III constitute the early matrix, followed by glycosaminoglycans and proteoglycans and collagen type I which compose the final matrix. The highest collagen amount in the wound is reached already after a couple of weeks post injury while the strength of the scar continues to increase for several months. Scar remodeling results in 6-12 month in a mature, avascular, and acellular normal scar. During normal tissue homeostasis, as well as in the healing wound, there is a constant turnover of collagen in the extracellular matrix. This is the result of collagenase activity. The synthesis and lysis of collagen are also controlled by cytokines and growth factors. TGF-β increases new collagen transcription and also decreases collagen breakdown by stimulating synthesis of tissue inhibitors of metalloproteinase (9) (10). This balance of collagen deposition and degradation is an important determinant of wound strength and integrity.

Epithelialization

The external barrier of the wound is restored by proliferation and migration of epithelial cells adjacent to the wound. This begins as early as within one day of injury. Basal cells at the edge of the wound lose their attachment to the underlying dermis and migrate across the surface of the provisional matrix. Fixed basal cells near the cut edge undergo mitosis and appear to migrate by moving over one another in a leapfrog fashion until the defect is covered (11). As soon as the defect is closed the migrating epithelial cells become more columnar in shape and increase their mitotic activity. The normal structure of the epithelium is re-established, and the surface layer eventually keratinizes. The stimuli for re-epithelialization remain unclear. However, it appears that the process is mediated by a loss of contact inhibition, exposure to constituents of the extracellular matrix, particularly fibronectin, and cytokines produced by immune mononuclear cells (12). In particular EGF, TGF-β, basic fibroblast growth factor (bFGF), PDGF, and IGF-1 have been shown to promote epithelialization.

SCARRING

Cutaneous scarring can be defined as a macroscopic disturbance of the normal skin structure and function as a result of a healed wound (13). Skin scarring might lead to prominent clinical problems. In addition to being disfiguring and aesthetically unpleasant scars can cause contraction, severe itching, tenderness, pain, sleep disturbance, anxiety, depression and disruption of daily activities. The clinical appearance of scarring covers a wide spectrum. Wound healing can result in a “normal” fine line scar or in a variety of abnormal scarring including widespread (stretched) scars, atrophic scars, scar contractures, hypertrophic scars and keloid scars. Scarless wound healing occurs only in early mammalian embryos and complete regeneration occurs in lower vertebrates, such as salamanders and invertebrates.

NORMAL SCARS

In scarring the amount of inflammatory cells, endothelial cells and fibroblasts decrease as the healing proceeds. The collagenous matrix becomes more organized into thicker and more cross-linked bundles indicating the development of the mature scar. Proteases and protease inhibitors such as plasmin, matrix metalloproteinases (MMPs), hyaluronidase and elastase are involved in extracellular matrix (ECM) remodeling (14) carefully controlled by growth factors, TIMPs and syndecans (15). The remodeling of the scar continues until at least 2 years after wounding. Scars are histologically characterized by a flattened epidermal-dermal junction and thickened epidermis. The collagen is abnormally organized into parallel bundles instead of the basket-weave organization in normal skin. In addition, the collagen fibres in scars are smaller and show a higher density in packing. Hair follicles and sebaceous glands never regenerate in a scar.

HYPERTROPHIC SCARS

Hypertrophic scars are raised scars remaining within the boundaries of the original wound (Figure 5). They generally regress spontaneously within month to years after the initial injury (16). Hypertrophic scars are often red, inflamed, itchy and even painful. They typically appear after burn injury on the trunk and extremities. Hypertrophic scars are frequently misdiagnosed as keloids (Table 1). Their gross appearance is similar, although keloids grow beyond wound margins.

Figure 5. Presternal hypertrophic scar after surgery.

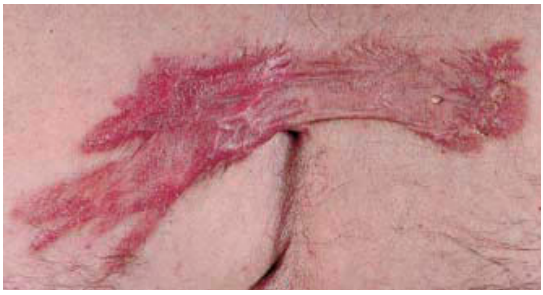

Figure 6. Typical presternal keloid. The margins are erythematous, growing into the surrounding healthy skin in a claw like appearance while the centre of the lesion has flattened and whitened.

KELOIDS

Keloids represent a pathological response to cutaneous injury resulting in disfiguring scars with no known satisfactory treatment (Figure 6). Skin injuries as burning, inflammation, surgery or minor trauma as insect bite induce an overabundant ECM deposition, especially collagen (17). Even the spontaneous development of keloids has been discussed (18) but may be the result of a minor, overlooked trauma.

The term keloid is derived from the Greek “khele”, for crab claw (19). Keloids have been mentioned for the first time in 1700 bc. in the “Smith-Papyrus” (20). In 1806 Alibert gave the first clinical description of keloids (21).

Keloids are benign skin tumours unique to humans. Clinically, keloids extend beyond the boundaries of the original wound and are seen predominantly in darker-pigmented individuals (22). Excessive or pathological scar formation is leading to devastating consequences for the patients such as contractures, pain, itching, paresthesia and psychological disorders. The exact pathogenic mechanisms underlying keloids continue to be elusive. Recently, research focus has been aimed at the biomolecular pathways responsible for the excessive ECM deposition.

Clinical characteristics and treatment

The differential diagnosis of keloids and hypertrophic scars is sometimes difficult. Keloids grow beyond the confines of the original wound, invading the normal surrounding skin and rarely regress over time. They often arise immediately after skin injury and appear as firm nodules which are pruritic and painful. Initially, keloids have a pink or red appearance and telangiectasias may be present (Figure 7). Keloids continue to grow, unlike hypertrophic scars, which typically reach a certain size and stabilize or regress (23) Strict clinical and histopathological criteria has been defined to differentiate keloids from hypertrophic scars (Table 1).

Of unknown reasons, keloids occur more frequently on the chest, shoulders, upper back, back of the neck and earlobes (Table 1) (24). In one case a massive 1.8 kg keloid has been removed from the arm at the site of vaccination (25). It has been intensively discussed whether keloids occur primarily in areas of high tension (26). This might be an oversimplification since the most commonly affected site, the earlobe (27), is under minimal tension and keloids appear rarely on the palms or soles where significant skin tension is to be expected.

Keloids	Hypertrophic Scars
grow beyond the borders of the original wound	remain within the boundaries of the original wound
size varies between a peach and a football	size often only a few centimeters
pruritic and painful	less pruritic and painful
firm, erythematous nodule	firm, erythematous nodule
appear within several months after initial scar, then gradually proliferate indefinitely	generally arise within 4 weeks, grow intensely for several months, then regress often within one year
occur often on the chest, shoulders, upper back, back of the neck and earlobes, rarely on the palms or soles	no predominant anatomical site
do not regress spontaneously in general	generally regress spontaneously after the initial injury
larger, thicker and more wavy collagen fibres than normal skin, random collagen fibre orientation, increased ratio of type I to type III collagen	fine collagen fibres oriented parallel to the epidermis
increased fibroblast density and fibroblast proliferation rate	increased fibroblast density
only few α-smooth muscle actin expressing myofibroblasts	presence of α-smooth muscle actin expressing myofibroblasts is typical

Table 1. Clinical and histological criteria's of keloids and hypertrophic scars (28).

The treatment of keloids is difficult and often unsatisfactory. Several treatment modalities have been tried, but most of them have rendered disappointing results. The most commonly used treatments include intralesional steroid injection, interferon, 5-fluorouracil, imiquimod and laser therapy. Primary excision and cryosurgery are among the major surgical options. Radiation therapies and other physical modalities such as silicon gel sheeting and pressure therapy are possible alternatives (29).

Epidemiology

Keloids can develop at every age but have a higher incidence between 10 to 30 years. Mean age of first keloid diagnosis is 22.3 years for women and 22.8 years for men (30). Hormones have been suggested to influence keloid formation supported by data showing an elevated androgen receptor level in clinical active keloid tissue (31). Keloids seem to have a higher incidence during pregnancy and puberty, which has been interpreted as related to the hormone profile but other explanations as increased neo-angiogenesis in pregnancy are possible (32). More studies are needed to confirm a correlation between keloid development and hormone profiles. Although epidemiologic data are limited they suggest differences among racial groups with a higher frequency in blacks, Hispanics and Asians. The incidence of keloids in the United Kingdom is reported to be less than 1% (33), while the incidence in blacks and Hispanics varies from 4.5 to 16% (32). The higher rate for earlobe piercing may be responsible for a slight female predominance for keloids (29).

Genetics

There is a familial predisposition to keloid scarring. TGF-β has previously been implicated in keloid pathogenesis. Thus, we tested for an association between keloid development and polymorphisms within the TGF-β1, -β2, -β3 and TGF-β receptor genes. Our studies do not reveal a significant association between TGF-β polymorphisms and keloid disease (34-37). To date, no susceptibility genes or gene groups have been identified. Marneros et al. provide the first genetic evidence for a keloid susceptibility loci and this might serve as a basis for the identification of responsible genes. He studied two families with an autosomal dominant inheritance pattern of keloids and identified linkage to chromosome 2q23 and chromosome 7p11 (38). Further, an association with the HLA-types HLA-DR5 and HLA DQw3 is proposed (39) and an autosomal dominant inheritance with incomplete penetrance has been suggested (40).

The association of keloids in certain syndromes like the Rubinstein-Taybi syndrome and Goeminne syndrome indicate a possible genetic background for keloids (41,42). The Rubinstein-Taybi syndrome is characterized by brachydactyly, facial abnormalities, and mental retardation while the Goeminne syndrome comprises congenital muscular torticollis, multiple keloids, cryptorchidism and renal dysplasia.

Immunology

Immunological reactions are likely to be involved in keloid etiology. Rossi et al. show a significant increased concentration of IgG in keloid lesions (39) and IgA and IgM are detected at higher levels in keloids compared to normal skin (43,44).

The immune cell infiltrate in keloids includes T lymphocytes (CD3+, CD4+, CD45RO+, HLA-DR+) and dendritic cells (CD1a+, CD36+, HLA-DR+, ICAM-1+) (45). The number of macrophages, epidermal Langerhans cells and mast cells are increased as well (46,47). The number of mast cells and pruritus decrease in keloids after treatment with silicone gel sheets and mast cells may contribute to an elevated expression of hypoxia-inducible factor 1, alpha (HIF-1alpha) and VEGF in keloids (48,49). The role of the inflammatory response in the formation of keloids has not been studied in details and remains to be elucidated.

Pathogenesis

The pathogenesis of keloids is poorly understood. No keloid-causing gene mutation has been reported so far and the *in vitro* experiments with keloid derived cells have revealed multifaceted and intricate pathological alterations in many different aspects of cell behaviour.

At present a single unifying hypothesis for the pathogenesis of keloid formation has not been put forward. Recent research has focused on the interaction between keloid derived fibroblasts and ECM, the role of integrins and the abnormalities of keloid fibroblasts (KF). In addition, the involvement of proteolytic remodeling of ECM by MMPs and plasmin and their inhibitors have been analyzed and several current studies have investigated the expression of cytokines, chemokines, growth factors and their signaling pathways in keloids.

Teofoli et al. find an altered TGF-β regulation of propiomelanocortin (POMC) gene expression in keloid-derived fibroblasts, suggesting that POMC may play a role in the pathogenesis of keloid formation (50). In this respect, it is interesting to note that so far no human albino with keloids has been described.

Keloids and ECM
The wound healing process requires a complex interaction between different cell types, ECM, and cytokines (51). A tightly regulated balance between synthesis and degradation of ECM is essential for normal scar formation (Figure 7). If this balance shifts towards increased ECM production or decreased degradation hypertrophic scars and keloids may occur.

In vitro, keloid fibroblasts show an elevated gene expression for collagen, fibronectin, elastin and proteoglycan (52-54). The gene expression of keloid fibroblasts is altered by the interaction with the surrounding ECM via cell surface receptors called integrins (55) and fibroblasts surrounded by densely packed collagen express different genes compared with fibroblasts in a softer matrix (56).

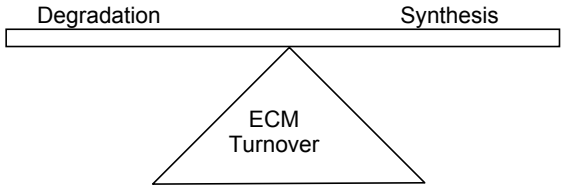

Figure 7. Regeneration and homeostasis of ECM requires a dynamic balance between synthesis and degradation to achieve optimal wound healing.

The balance between ECM degradation and synthesis is influenced by proteolytic processes. This remodeling of the granulation tissue in the last phase of wound healing replace collagen type III with type I, increase proteoglycan production and degrade fibrin and fibronectin. The ECM remodeling is mediated by proteolytic serine proteases (tissue-plasminogen activator (t-PA) and urokinase-plasminogen activator (u-PA)) and MMPs produced by fibroblasts. MMP-1 (collagenase-1), MMP-8 (collagenase-2) and MMP-13 (collagenase-3) are all capable to cleave collagen type I, II and III (57).

Another regulator involved in keloid development is the plasmin/plasminogen activator system. U-PA and t-PA activate plasminogen to plasmin which acts fibrinolytic and activates procollagenase (58). Further, plasmin is involved in activating TGF-β from its latency form (59) (Figure 8) and TGF-β induces plasminogen activator inhibitor-1 (PAI-1) and TIMP-1 leading to decreased plasmin and collagenase activity resulting in diminished collagen degradation.

Keloid fibroblasts express higher levels of PAI-1 (60) leading to increased accumulation of collagen and fibrin (61). An alternative or additional reason for elevated PAI-1 expression might be hypoxia and an increased level of the hypoxia marker HIF-1α is found in keloid tissue (62).

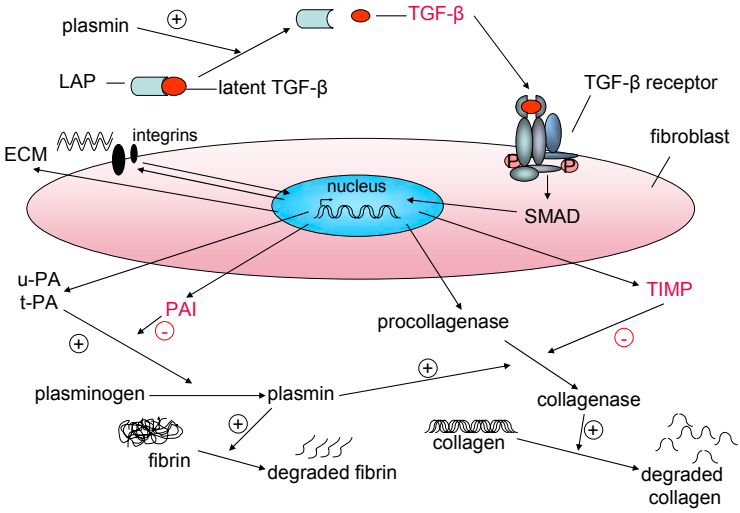

Figure 8. Feed-back loop between fibroblasts, TGF-β, ECM and other soluble factors in wound healing (modified from Tuan et al. 1998)

Integrins

The contact of fibroblasts with the surrounding ECM is established mainly by integrin receptors. These are transmembrane receptors, which specifically bind different ECM molecules. A group of five integrins, α1β1, α2β1, α3β1, α10β1 and α11β1 have been described as binding to collagens, with some members also binding to other ECM molecules like laminin (α1β1) or fibronectin (α3β1). On fibroblasts, recognition of external collagen is mainly achieved by α1β1 and α2β1 integrins. On the collagen side, the binding epitope needs to be in a native, i.e. triple-helical conformation; within the integrin receptor, the major collagen binding site resides in the A-domain (also called I-domain) of the respective α1 or α2 subunits (56). Integrin expression is regulated by cytokines as for example TGF-β in an autocrine and paracrine manner (55). This allows a change in affinity of integrins towards their ligands (63). Keloid fibroblasts may be able to recognize the changed composition of the surrounding ECM by integrins. This may change the phenotype of keloid fibroblasts augmenting keloid development.

Keloids as well as hypertrophic scars show alterations in integrin expression and contain several distinct populations of fibroblasts. One of these populations expresses high levels of α1 integrin, and the proportion of these cells is higher in keloids and hypertrophic scars than in normal skin tissues. Integrin α1 knockout mice maintain increased collagen synthesis consistent with a role for α1 integrin in providing negative feedback on collagen synthesis (64). These results from mice are controversial. A down-regulation of α1 integrin in keloids would be expected but the high expression of TGF-β in keloids maintain high levels of α1 integrin and up-regulated collagen production.

Keloid fibroblasts

Most *in vitro* studies investigate the ECM-producing keloid fibroblasts. Keloid fibroblasts show an increased production of fibronectin and type I procollagen (65). Their ability to degrade procollagen polypeptides is reduced (66) compared to normal dermal fibroblasts. In addition, keloid fibroblasts have a reduced growth factor requirement for proliferation (67) and the growth response to EGF is significantly increased (68). TGF-β1 stimulation leads to an altered response of the total protein synthesis (69) and induces up-regulated collagen production (70).

Several studies show a different cytokine expression pattern in keloid fibroblasts. Their expression of TGF-β1 and β2 is elevated (71). Keloid fibroblasts are more responsive in both chemotactic and mitogenic assays to all three isoforms of PDGF than fibroblasts from normal skin. No enhanced response to either EGF or FGF is detected. The enhanced PDGF response of keloid fibroblasts appears to be mediated by elevated levels of PDGF α receptors, which are 4-5 times higher than those in normal human skin fibroblasts (72). Wu et al. show that VEGF is expressed at higher levels in keloid tissues and their derived fibroblasts compared with normal skin and VEGF stimulates the expression of PAI-1, but not u-PA in keloid fibroblasts (73). These findings suggest that VEGF may play an important role in keloid formation by altering ECM homeostasis towards a state of impaired degradation and excessive accumulation.

There is evidence that keloid fibroblasts fail to undergo physiologically programmed cell death (apoptosis). They are more resistant to Fas-mediated apoptosis (74) and the overexpression of IGF-1 receptor inhibits ceramid-induced apoptosis (75). Sayah et al. demonstrate underexpression of pro-apoptotic genes (tumor necrosis factor receptor 1-associated protein (TRADD); 19-kDa interacting protein 3 (NIP3); and cytoplasmic dynein light chain 1 (HDLC1)) in human keloid tissue and decreased apoptotic activity in fibroblasts derived from keloids versus those from normal scars (76). Keloid fibroblasts show a focal dysregulation of p53 combined with upregulation of bcl-2 which facilitate increased cell proliferation and decreased cell death (77).

Recent studies reveal altered interactions between keratinocytes and fibroblasts in keloids. Co-culture of keloid fibroblasts with keloid keratinocytes induces greater resistance of fibroblasts to apoptosis, increases TGF-β and collagen type I and III expression compared to keloid fibroblasts from pure cultures (78,79). Interestingly, co-culture of normal fibroblasts with keloid keratinocytes leads to increased proliferation of fibroblasts and increased TGF-β and collagen type I and III expression (80).

TGF-β and SMAD

TGF-β family members, which include TGF-β, activin, and bone morphogenetic protein (BMP), are secreted cytokines that regulate a broad array of cellular responses including proliferation, differentiation, migration and apoptosis. Dysregulation of their signaling has been implicated in various human diseases including cancer, fibrosis, autoimmune diseases and vascular disorders. Several growth factors are involved in wound healing but TGF-β appears to play a central role (81). Most cells implicated in wound healing secret TGF-β. Three mammalian isoforms of TGF-β exists: TGF-β1, TGF-β2 and TGF-β3 (82).

Quite a few mechanisms exist to regulate the expression of TGF-β: TGF-β activity is regulated by TGF-β gene transcription. TGF-β1 itself up-regulates the expression of TGF-β1 mRNA and protein (83). The active form of TGF-β is non-covalently bound to the latency-associated peptide (LAP) (84) and subsequent activation is required to release TGF-β from its latent form. In addition, the latent TGF-β forms a larger complex with a protein called latent TGF-β-binding protein (LTBP). This complex binds on the cell surface where the LAP region binds to mannose-6-phophate (IGF2) receptors. Transglutaminase initiates cross-linking of plasminogen to the cell surface which turns into plasmin and releases active TGF-β (85). The availability of TGF-β is also regulated by the ECM. Decorin, α-fetoprotein and biglycan bind and neutralize TGF-β (86,87) and fibronectin and thrombospondin associated TGF-β exhibit biological activity (88,89).

The TGF-β receptors consist of three subtypes, type I, type II and type III. They belong to the serine/threonine kinase family of receptors and have an extracellular domain, a transmembrane domain and a cytoplasmatic kinase domain. TGF-β1 binds first to the type II receptor (TβRII) and then TβRI is recruited to the complex (90). TβRII phosphorylates TβRI to activate it. The affinity of TGF-β2 to TβRII is low and requires TβRIII for assembly but the role of TβRIII is not fully clarified. TβRIII exists also in a soluble form found in serum and in ECM which might act as a reservoir or clearance system for bioactive TGF-β or facilitate TGF-β binding to its receptors (91).

TβRI initiate intracellular signaling by phosphorylating specific proteins known as SMAD proteins (for Sma and Mad proteins from Caenorhabditis elegans and Drosophila, respectively) (92). SMAD proteins can be divided into three classes: the receptor-activated SMADs (R-SMADs), the common-mediator SMADs (Co-SMADs), and the inhibitory SMADs (I-SMADs).

SMAD proteins share two highly conserved domains, Mad-Homology domains 1 and 2 (MH1 and MH2) at N- and C-terminal parts of the proteins. R-SMADs transiently interact via their MH2 domains with TβRI and become phosphorylated at their C-terminal (93). SMAD1, 5 and 8 are involved in bone morphogenetic protein (BMP) signaling. SMAD2 and 3 are restricted to the TGF-β pathway (94).

Upon activation, R-SMADs (SMAD2/3) form heteromeric complexes with Co-SMADs (SMAD4) via their MH2 domains (Figure 9). This complex accumulates in the nucleus, where it participates in the control of expression of target genes. Inhibitory SMADs (SMAD6 and 7) prevent the activation of signal-transducing R- and Co-SMADs. SMAD6 seems to preferentially inhibit BMP signaling and SMAD7 seems to be more specific for TGF-β signaling (94-96). SMAD6 and 7 interact efficiently with activated TβRI and compete with R-SMADs for binding to the activated TβRI (97). As the expression of inhibitory SMADs is induced by ligand stimulation (98), they may have a negative-feedback role in signal transduction and thereby forming another regulatory pathway for TGF-β activity.

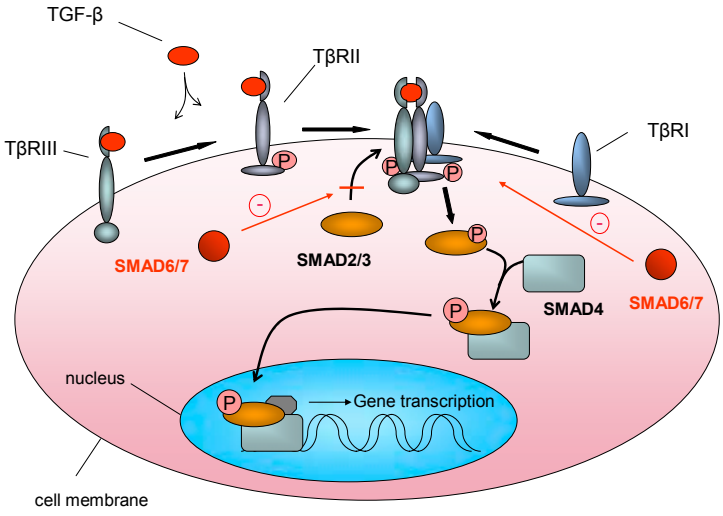

Figure 9. Model of intracellular TGF-β signaling events. (TβRI/II/III, TGF-β receptor I, II and III)

TGF-β has a large variety of biological effects in wound healing. It acts as a potent chemoattractant, it induces proliferation and angiogenesis and stimulates ECM synthesis as well as prevents its degradation by up-regulation of tissue inhibitors of metalloproteinases (TIMPs) and down-regulation of proteases.

An abnormal production of TGF-β has been shown to have devastating effects. For example, TGF-β seems to be responsible for the excessive ECM deposition in fibrotic diseases as bleomycin-induced pulmonary fibrosis (99), idiopathic pulmonary fibrosis (100) and systemic sclerosis (101). Neutralizing TGF-β antibodies prevent increased ECM deposition in experimental glomerulonephritis (102) and reduce the rate of collagen synthesis of keloid fibroblasts (103). In line with these findings Shah et al. injected neutralizing TGF-β antibody to the margins of healing dermal wounds in adult rats and found wound healing without scar-tissue formation (8). These results point towards a possibility that early manipulation of selected cytokines may be a successful approach to the control of scarring.

QUALITY OF LIFE

Patients with keloids and hypertrophic scars avoid visiting public swimming pools due to their stigmata. Having a stigma and being excluded from full social acceptance is a central problem for all persons who are ‘‘different’’ as compared to ‘‘normal’’ (104). Beside the clinical manifestation, psychological measures, especially quality of life are very important parameters in determining the efficacy and acceptance of disease treatments (105,106). As the treatment of keloids is often difficult and frustrating, the development of a disease-specific questionnaire designed to measure quality of life in patients with keloids and hypertrophic scars is highly desirable.

The World Health Organization developed the concept of quality of life (QOL) from a wide spectrum of information about physical, social and psychologic well-being. Health is not only the absence of disease (107) but the ability to live a productive and enjoyable life. QOL questionnaires can be used to evaluate or compare the complex effects of an intervention or treatment on health (108). QOL measurement is based on informations from the patient’s perspective about the impact of disease on daily living and offers a systematic method for evaluating the benefits of treatment in terms of what patients value (109).

The objective assessment of skin disease severity does not necessarily reflect the effects of psychological stress, embarrassment and stigma (110). Therefore, numerous investigations have been performed to assess the quality of life of patients with chronic skin diseases like psoriasis and atopic dermatitis (111-114). Rapp et al. demonstrate that the quality of life of psoriasis patients is as much reduced as in patients with severe heart failure or diabetes mellitus (115).

With the exception of some quality of life studies in patients with burn scars such investigations are rare in patients with pathological scarring (116-122). In keloid and hypertrophic scarring, there is still no prospective analysis using validated instruments in a large cohort of patients. Furthermore, keloids and hypertrophic scars are not often properly discriminated from various clinical phenotypes of normal scarring. Clinical experience suggests that patients with keloid and hypertrophic scarring suffer as much as patients with other chronic skin diseases and that this impairment in the quality of life is influenced mostly by internalized and experienced stigmatization. The recognition of stigmatization plays a central role in patients with skin diseases and is often more important to the patient than the physical disease itself (123,124) as for example the impact of disease severity on quality of life in vitiligo or psoriasis (125,126).

AIMS OF THE THESIS

GENERAL AIM

The general aim of the thesis is to gain deeper insight into the role of TGF-β and SMAD proteins in the pathogenesis of keloids, to describe the quality of life of patients with keloids and to analyze gene expression patterns in different keloid parts.

SPECIFIC AIMS

- To investigate the expression of TGF-β1, TGF-β2 and TGF-β3 and their receptors TβRI and TβRII in keloids.
- To study the effect of TGF-β1 stimulation on TβRI and TβRII expression in keloids.
- To examine the expression of SMAD2, 3, 4, 6 and 7 in keloids.
- To investigate the effect of TGF-β1 stimulation on SMAD expression in keloids.
- To develop a questionnaire to analyze the quality of life of patients with keloids.
- To clarify if genes are differentially expressed in different parts of keloids.
- To identify genes which might be targets for future treatment strategies.

MATERIALS AND METHODS

CELL CULTURE (PAPER I, II and IV)

As one problem associated with studies in keloid patients is a proper definition of keloids and hypertrophic or normal scarring, the selection of patients is performed by experienced dermatologists. We adapt strict clinical criteria (keloids are dermal tumours that spread beyond the margin of the original wound, continue to grow over time, do not regress spontaneously, commonly recur following excision) to identify keloid cases as opposed to hypertrophic scars and in addition we require the presence of keloids for at least a minimum period of 1 year. Healthy Caucasian keloid cases (18-65 years of age) are enrolled in the study. None of the patients are smokers and none receive oral medication. Age-, gender- and site-matched skin from healthy donors, normal scars, hypertrophic scar patients and keloid patients are obtained in the same way. In paper I and II, biopsies are taken from the active margin of keloids. Primary human fibroblast cultures are prepared from fresh tissue punch-biopsies (5 mm diameter). In paper I TGF-β and TGF-β receptor mRNA expression is analyzed in subconfluent fibroblast cultures from keloids (n=5), hypertrophic scars (n=5) and control skin (n=5). In paper II SMAD mRNA and protein is analyzed in subconfluent fibroblast cultures from keloids (n=4), normal scars (n=3) and control skin (n=5). Fibroblasts are stored in T75 flasks (Nunc, Life Technologies Ltd, Germany). Monolayer-cultures are obtained in RPMI-1640 medium (Cell concepts, Umkirch, Germany) supplemented with 2 mMol/l L-glutamine, 100 U/ml penicillin and 100 U/ml streptomycin (Biochrom, Berlin, Germany), 10% heat-inactivated fetal calf serum (FCS; PAA Laboratories, Linz, Austria) and 25 mMol/l N-2-hydroxylpiperizine-N'-2-ethanesulphonic acid (HEPES) (Cell concepts). In paper IV three keloids from different donors are initially excised, subcutaneous tissue is carefully removed and the edges of the keloids are trimmed to remove unscarred tissue. 5 mm punch biopsies are than taken from three locations of the excised keloid tissue: From the deeper part of the keloid centre (A), the superficial part of the keloid centre (B) and from the superficial part of the active erythematous keloid margin (C) (Figure 10).

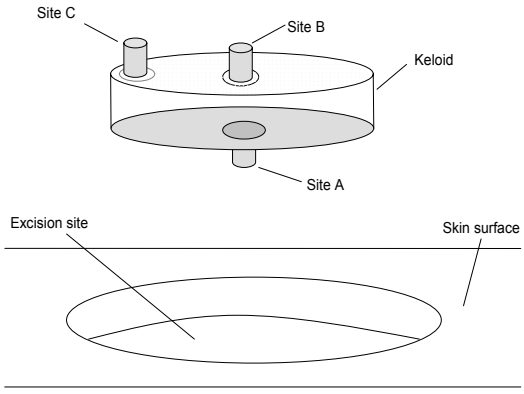

Figure 10. Schematic model of an excised keloid with the biopsy sites (A, B, C).

Control tissue is not allowed from keloid patients of ethical reasons. Therefore, 5 mm punch biopsies are taken from healthy skin of three different donors. Dermis from the biopsies is minced and incubated in a solution of collagenase type I (0.5 mg/ml) and trypsin (0.2 mg/ml) at 37 C for 6 h. Cells are pelleted and grown in the tissue culture flasks. Monolayer-cultures are obtained in D-MEM medium (Cell concepts, Umkirch, Germany). Cells are incubated at 37°C in a 5% (v/v) CO_2 humidified atmosphere. Cells from passages two to four are used for the experiments described. No cells are kept more than 4 weeks in culture. For experiments cells are trypsinated and transferred to phosphate-buffered saline (PBS).

STIMULATION OF CULTURED FIBROBLASTS WITH TGF-β1 (PAPER I and II)

Twenty-four hours before treating cultured fibroblasts with TGF-β1, the regular medium is replaced with serum free medium. After washing twice with PBS, sub-confluent fibroblasts are incubated with various concentrations of TGF-β1 (R&D Systems, Wiesbaden, Germany) diluted with 1% FCS (PAA Laboratories) in RPMI-1640 at different time points. Cells incubated with 1% FCS RPMI-1640 medium served as negative controls.

REAL-TIME REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION (RT-PCR) (PAPER I, II and IV)

Real-time RT-PCR analyses are performed in a fluorescence temperature cycler (LightCycler, Roche Molecular Biochemicals) according to the manufacturer's instructions. Briefly, total RNA from cultured fibroblasts is isolated using Trizol reagent (Invitrogen) and 2 µg of total RNA is reverse-transcribed using standard reagents (Invitrogen). The cDNA corresponding to 20 ng of RNA serves as a template in a 20 µl reaction containing 4 mM $MgCl_2$, 0.5 µM each primer, and 1x LightCycler-FastStart DNA Master SYBR Green I mixture (Roche Molecular Biochemicals). When the SYBR Green I dye intercalates into dsDNA, its fluorescence increases greatly (Figure 11).

Samples are incubated for an initial denaturing at 95 °C for 10 min, followed by 45 cycles, each cycle consisting of 95 °C for 15 sec, 60 °C (touchdown of -1 °C/cycle from 66 °C to 60 °C) for 5 sec, and 72 °C for 10 sec. Cycle-to-cycle fluorescence emission readings are monitored at 72 °C at the end of each cycle and analyzed using LightCycler Software (Roche Molecular Biochemicals). The software first normalizes each sample by detecting the background fluorescence present in the initial cycles. Then a fluorescence threshold at 5% of full scale is set, and the software determines the cycle number at which each sample reaches this threshold. The threshold fluorescence cycle number correlates inversely to the log of the initial template concentration. Relative transcript levels are corrected by normalization based on GAPDH levels. Melting curves are generated after each run to confirm amplification of specific transcripts. All quantifications are normalized to the housekeeping gene GAPDH that is amplified by intron-spanning primers. Standard curves are obtained for each primer set with serial dilutions of cDNA.

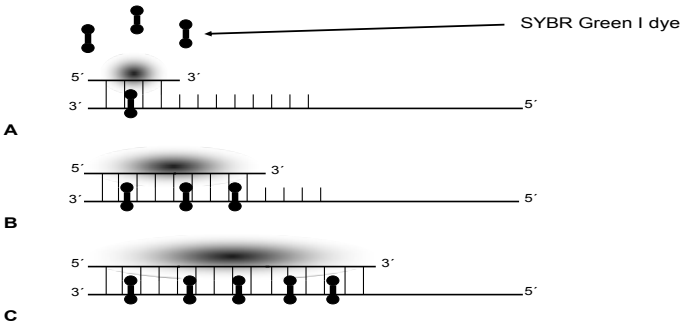

Figure 11. Monitoring real-time RT-PCR with SYBR Green I dye. During annealing (A), PCR primers hybridize to the target and form small regions of double-stranded (ds) DNA where SYBR Green I intercalates. In the elongation phase (B), more dsDNA is formed and more SYBR Green I dye can intercalate leading to a higher fluorescent signal. At the end of the elongation phase (C), all DNA has become double-stranded and the maximum amount of SYBR Green I is intercalated.

In paper IV, RNA is extracted using the RNeasy-kit (Qiagen, Hilden, Germany) and reversely transcribed with the High-Capacity cDNA Archive Kit (Applied Biosystems, Foster City, California). All reference mRNA sequences available from the NCBI Gene Bank are included in the search for possible primer binding regions. Primers are designed with Primer Quest software (Integrated DNA Technologies, Toronto, Canada). Specific primers are optimized for concentrations between 50 and 900 nM. The relative quantification real-time RT-PCR reaction for each gene is performed on 75 ng cDNA with 300 to 900 nM specific primers under the following conditions in the 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, California): Taq polymerase activation for 10 min at 95 °C followed by 40 amplification cycles of a 15 sec denaturation interval at 95 °C and a 60 sec annealing-step at 60°C with a single fluorescence measurement. Amplification of specific PCR products is detected using the SYBR Green PCR Master Mix (Applied Biosystems). Experiments are performed in duplicate in a total reaction volume of 20µl, using RPS9 as housekeeping gene for normalization. Regulatory effects observed by array analysis are confirmed by real-time RT-PCR for seven selected genes (Table 2).

RT-PCR	HAS1	STAT1	MMP3	THBS2	LIF	PTX3	IGFBP5

Table 2.

WESTERN BLOT ANALYSIS (PAPER II)

Western blotting identifies the presence and quantity of a specific protein (127). The proteins are transferred to a membrane using electrophoresis.

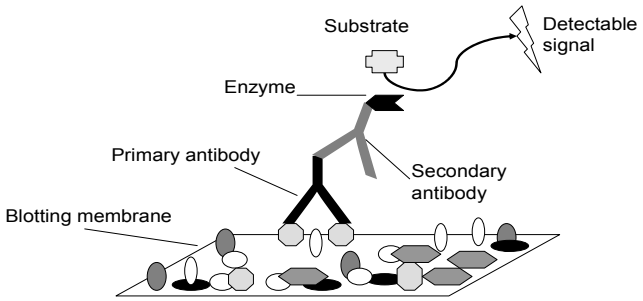

Figure 12. Immunodetection on Western blot membrane.

A specific primary antibody is used to detect the protein (Figure 12). An enzyme conjugated secondary antibody interacts with the primary antibody and then reacts with the substrate which generates a detectable signal.

To analyze SMAD2/3, 4, and 7 protein, sub-confluent cultured fibroblasts derived from keloids, normal scars and control skin are rinsed with PBS and then homogenized in RIPA buffer. The cell lysate is assayed for protein concentration by using Bicinchoninic acid (BCA) protein assay kit (Pierce, Illinois, USA). Aliquots of samples are then stored at -20 °C until further use. Equal amounts of protein samples (40 mg) are solubilised in Laemmli sample buffer and then separated on 10% SDS gels by SDS-PAGE. Rainbow protein molecular weight markers (Amersham, Braunschweig, Germany) are used to monitor protein separation. Proteins from the gel are electrotransferred onto a nitrocellulose membrane (0.45 mm) (Bio-Rad, Munich, Germany). To reduce unspecific binding nitrocellulose membranes are blocked overnight in a blocking buffer containing 5% skim milk powder (Merck) and incubated for 2 h at room temperature with primary polyclonal antibodies. Following incubation membranes are rinsed with washing buffer TBST. Membranes are then incubated for 1 h at room temperature with the secondary antibody conjugated with horseradish peroxidase (HRP) (Dianova, Hamburg, Germany). Western blots are visualised by peroxidase reaction using ECL Western blotting reagent (Amersham) over Kodak Scientific imaging film (Kodak, New York, USA).

To identify specific bands, cell lysates from a SMAD3 expression-vector-transfected MG63 cell line and SMAD7 expression-vector-transfected pancreatic carcinoma cell line (Panc1) as well as SMAD4 expression-vector-transfected Panc1 cell line are used as positive control. Immunoblottings are repeated at least three times with each sample with similar results. A dot blotting method is used to identify the optimal concentration of primary and secondary antibodies for immunodetection. The membranes are reprobed with β-actin antibodies to verify that an equal amount of the protein is loaded.

IMMUNOHISTOCHEMISTRY (PAPER I)

To study the localization and expression of TβRI and TβRII, punch biopsies (5 mm) are taken from keloids, hypertrophic scars and normal skin, immediately stored in 0.9% NaCl and snap-frozen in liquid nitrogen. Each biopsy is cut into 5 µm thin slices, fixed on glass slides and dried at room temperature overnight. The tissue is then fixed for 10 min using acetone. After fixation slides are incubated for 1 h with the primary antibody. Specific antibodies against TβRI and TβRII (200 mg/ml) (polyclonal IgG, rabbit, Santa Cruz) are used for staining at a dilution of 1:200. Following incubation each slide is thoroughly rinsed three times and incubated for 1 h with the secondary antibody (polyclonal anti-goat and antirabbit IgG, Santa Cruz). The immunohistochemical procedures are performed according to the labelled streptavidin–biotin method (LSAB, Dako, Carpinteria, CA, USA).

MICROARRAYS FOR EXPRESSION PROFILING (PAPER IV)

The use of microarrays for expression profiling is first published in 1995 by Schena et al. (128). A microarray is a solid substrate, such as a silicon wafer or glass slide, on which DNA or oligonucleotides are attached. These nucleic acids are complementary to thousands of genes (129).

There are two main types of microarrays: spotted DNA and oligonucleotide arrays. In our study, we use the oligonucleotide array. Oligonucleotides can be directly synthesized in situ on to the solid substrate. Each oligonucleotide is about 50-70 nucleotides long. Over the slide there are several different oligonucleotide sequences (approximately 20) from the same gene. This range produces more robust results because the same gene can be probed independently several times in the same experiment. This method - Affymetrix microarrays (Affymetrix, CA, USA) - allows greater specificity. For each nucleotide sequence there is a duplicate sequence with a single base change, called a mismatch. This allows comparison between hybridisation to a specific sequence and background hybridisation to non-specific sequence and true quantification.

Figure 13 shows the principles of the oligonucleotide array. After extraction RNA is reverse transcribed into double-stranded cDNA. Then, in vitro transcription and biotin labeling of cRNA is performed. cRNA is then fragmented and hybridized on to the chip. After hybridization cRNA is fluorescently labeled by adding streptavidin-phycoerythrin and scanned.

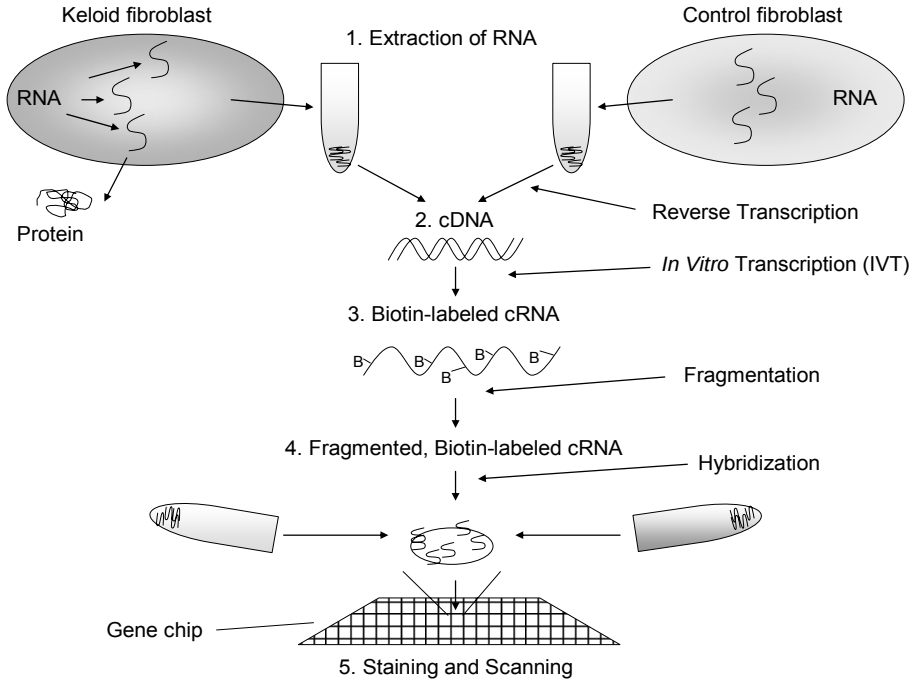

Figure 13. The principles of the oligonucleotide array.

LABELLING OF RNA AND ANALYSIS OF GENE EXPRESSION USING MICROARRAYS (PAPER IV)

To identify differentially expressed genes in different sites of keloids, keloid fibroblasts are grown to subtotal confluence in 75 cm² flasks. Purification and labelling of RNA is then performed as described previously (130). Briefly, in three independent experiments total RNA is extracted from keloid fibroblasts and normal skin fibroblasts. Equal amounts of total RNA, from each independent experiment, are pooled into three different pools. RNA is then converted to double-stranded cDNA using a modified oligo-dT primer with a *5´* T7 RNA polymerase promoter-sequence. Then, *in vitro* biotin-labeled cRNA transcription is performed using T7 RNA polymerase and the cDNA template in the presence of a mixture of unlabeled nucleotides (ATP, CTP, GTP and UTP) and biotin-labelled CTP and UTP (BioArray High Yield RNA Transcript Labeling Kit; Enzo Diagnostics, Farmingdale NY).

Biotin-labelled cRNA is then purified on microspin columns provided with the RNeasy Mini Kit from QIAGEN (Hilden, Germany) and fragmented to an average size of 100 – 150 bases according to the Affymetrix (Santa Clara, CA) protocol. The amount of labeled cRNA is determined by measuring absorbance at 260nm.

For each pool of cRNA two Affymetrix Human Genome U133 Plus 2.0 Arrays, covering the whole human genome, are hybridized. Hybridization is carried out at 45 °C for 16 h after which arrays are washed in an automated Affymetrix fluidic station. Hybridized cRNA is (128) fluorescently labeled by adding streptavidin-phycoerythrin (Molecular Probes, Eugene, OR). Stained arrays are then scanned in a Hewlett-Packard Gene Array Scanner (Agilent Technologies, Palo Alto, CA).

Data is analyzed using Affymetrix Microarray Suite 5.0. Using the Affymetrix Data Mining Tool (DMT), gene expression is evaluated and genes with at least a 2-fold or greater change in signal intensity in 2 independent experiments are considered regulated. In the DMT, p-values are set to less than 0.001 for up-regulated genes and set to more than 0.999 for down-regulated ones.

ENZYME-LINKED IMMUNOSORBENT ASSAY (PAPER IV)

Engvall and Perlman (131) are one of the first to describe the enzyme-linked immunosorbent assay (ELISA). Several different techniques of this immunoassay have been developed since then. However, the sandwich-type ELISA is one of them with a higher sensitivity in which standards, controls and protein samples are incubated in microtitration wells which have been coated with the specific primary antibody. After incubation, the wells are treated with another secondary antibody labelled with an enzyme. The degree of enzymatic turnover of the substrate is determined by photometric measurement. The absorbance measured is directly proportional to the concentration of the protein present in the sample.

Inhibin beta A (INHBA) is measured in conditioned cell culture media samples of normal skin fibroblasts and keloid fibroblasts using a specific ELISA according to the manufacturer's instructions (INHBA, Oxford Bio-Innovation, Upper Heyford, UK). Signal transducer and activator of transcription 1 (STAT1, Calbiochem, Darmstadt, Germany) is measured in cell pellet lysates of normal skin fibroblasts and keloid fibroblasts. Monocyte chemoattractant protein-1 (MCP1, eBioscience, San Diego, California) and activated matrix metalloproteinase 3 (MMP3, Ray Biotech, Norcross, Georgia) are measured in tissue culture supernatants of normal skin fibroblasts and keloid fibroblasts. Tissue culture supernatants and cell pellets from 3 independent experiments are used.

DEVELOPMENT OF THE QUALITY OF LIFE QUESTIONNAIRE (PAPER III)

To describe and measure the quality of life of patients with keloids and hypertrophic scars a new questionnaire is developed. First, items are selected with regard to the physical and psychological problems associated with these diseases. An item pool is formed by screening the items of the questionnaire on Experience with Skin Complaints (112,113) concerning their relevance for patients with scars. In a second step, these items are semantically modified. After this, a pilot study is conducted on ten outpatients presenting the selected items in a preliminary questionnaire. Items are graded in six steps (-5=totally inaccurate, -3=inaccurate, -1=somewhat inaccurate, 1=fairly accurate, 3=accurate, 5=completely accurate).

This scaling is chosen to make sure that there is the same distance (-2 and 2) between each step (‘‘requirement of equidistance’’).

The study population is a result of consecutive examination of outpatients seen in a “Scar-Service clinic “at the Department of Dermatology, University of Kiel, over a period of 11 months. In order to determine reliability, this questionnaire is handed out at the first examination and 2 weeks later (2-week test–retest method). Lesions of the patients are classified as keloids or hypertrophic scars and discriminated from normal scars by specialists in the field. The number of scars and the regions of the body affected are documented using a specialized proforma.

First results regarding validity are be obtained by comparison with clinical findings. In addition, single items measured subjective intensity of and suffering from disease (graded as six steps from very slight/none to very severe). Different aspects clinically characterizing the disease (pruritus, intensity of scar pain, decrease of mobility) are measured on a visual analogue scale from 0 to 10. Finally, basic sociodemographic data are gathered.

STATISTICAL ANALYSIS (PAPER I, II, III and IV)

In paper I and IV statistical significance between groups is analyzed by Student’s unpaired t-test. All values are expressed as mean ± SD. Statistical significance is defined as $p \leq 0.05$. In paper II statistical significance between groups is analyzed by one way analysis of variance (ANOVA) followed by Turkey multiple comparison test post-analysis to evaluate statistical differences among samples.

In paper III a factor analysis (maximum-likelihood procedure, varimax rotation) of 34 items is performed to analyze the underlying latent constructs of this item pool. The scree-criterion could not be interpreted definitely, suggesting use of a two- or three-factor solution. However, two dimensions of the three-factor solution representing different aspects of the psychological impairment induced by keloid and hypertrophic scarring, that is, intrapsychic point of view and interpersonal relationships, show high correlation. Therefore, the two-factor solution is chosen corresponding to a distinct content of each factor (‘‘psychological impairment’’ and ‘‘physical impairment’’). A binomial test is performed to determine the deviation of the sex relation in this sample from the expected relation (‘‘true prevalence’’). Relations between the two scales of this questionnaire to relevant sociodemographic (e.g., age, gender), disease related (e.g., duration, pruritus, pain, restriction of mobility due to disease) and psychosocial parameters (e.g., visibility, suffering from disease) are determined by the ‘‘Pearson-correlation.’’ The data are analyzed using the statistics program system SPSS.

ETHICAL CONSIDERATION

The first three studies are performed after approval of the Ethical Committee of the Kiel University Hospital and with the signed written consent of patients and controls. Study four is approved by the Ethical Committee at the University Hospital Linköping.

Patients consent is obtained following oral and written information. All four studies follow the Helsinki declaration (http://www.wma.net/e/policy/b3.htm) and following amendments by the World Medical Association.

RESULTS

PAPER I

In this study, we investigate the mRNA expression of TGF-β and its receptors in cultured human fibroblasts of keloids, hypertrophic scars and control skin.

The expression of TGF-β1 mRNA is significantly increased in cultured keloid fibroblasts compared to hypertrophic scar and control skin fibroblasts. Hypertrophic scar fibroblasts show a significantly decreased expression of TGF-β2 mRNA compared to keloids and control skin. TGF-β3 mRNA is found to be significantly down-regulated in cultured fibroblasts from keloids compared to hypertrophic scars and control skin.

In addition, we determine the expression levels of TGF-β receptor type I (TβRI) and TGF-β receptor type II (TβRII) mRNA in the same cell lines. Hypertrophic scar derived fibroblasts reveal a significantly down-regulated expression of TβRI mRNA compared to keloids and control skin. The expression of TβRII mRNA shows a significantly reduced expression in keloid derived fibroblasts and the ratio of TβRI and TβRII mRNA expression is significantly higher in keloid derived fibroblasts. Figure 14 gives an schematic overview of the results obtained in keloid and control skin fibroblasts. We hypothesize that the homeostasis in ECM production and degradation in control skin depends on the proportion of TGF-β subtypes and receptors shown in Figure 14. Changes in this proportion as seen in keloids lead to increased ECM production and reduced ECM degradation. This means, that increased TGF-β1, increased ratio of TβRI/TβRII in combination with decreased TGF-β3 may be involved in keloid development.

To analyze the effect of TGF-β1 stimulation on TGF-β receptor expression, cells are exposed to TGF-β1. TGF-β1 stimulation induces slight up-regulation of TβRI and TβRII in hypertrophic scar derived fibroblasts. In contrast, TGF-β1 induces a non-significant down-regulation of TβRI and TβRII in keloid and control skin derived fibroblasts.

To investigate the expression of TβRI and TβRII *in vivo*, immunohistochemistry of tissue biopsies of keloids, hypertrophic scars and control skin is performed. The whole epidermis shows a slight positive staining for TβRI and TβRII increasing from the basal cells to the uppermost parts of the epidermis. Positive staining for TβRI and TβRII in the dermis is found in hair follicles and fibroblasts in keloids, hypertrophic scars and control skin. The staining pattern of TβRI and TβRII did not differ between keloids, hypertrophic scars and control skin.

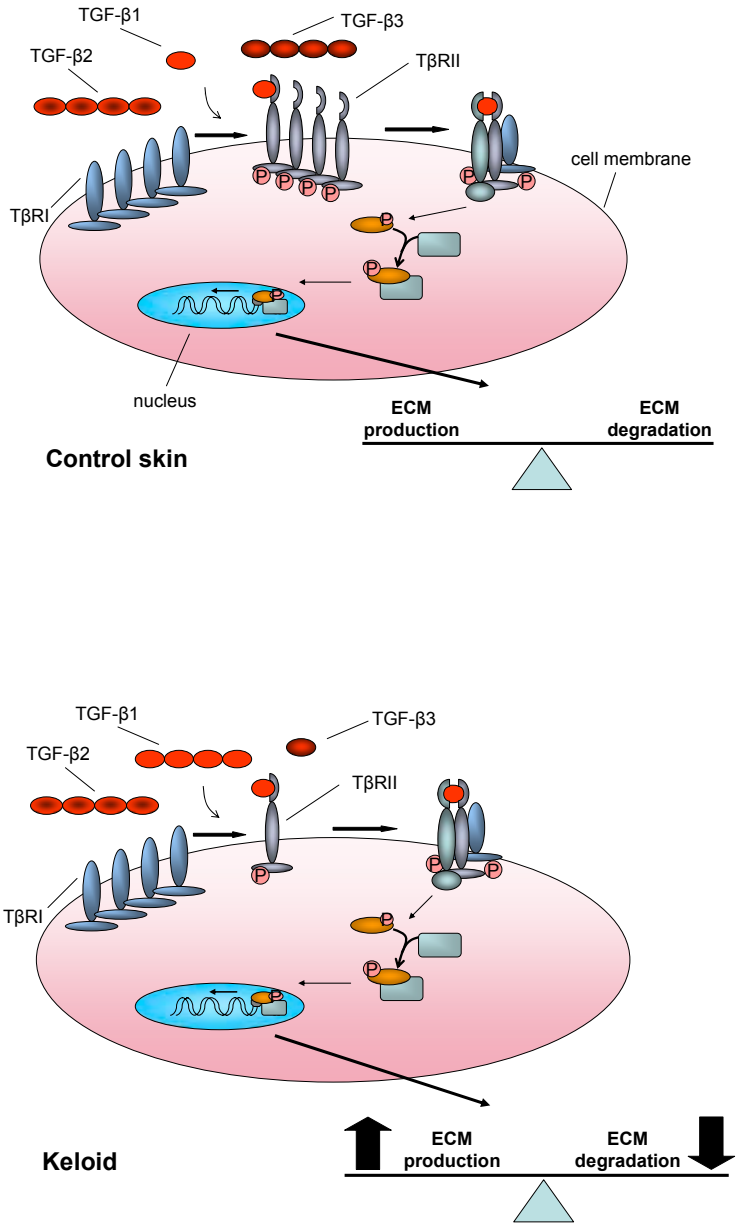

Figure 14. Proposed model for the fibrotic capacity of specific TGF-β and TGF-β receptor relations. (ECM, extracellular matrix; TβRI/II, TGF-β receptor I and II)

PAPER II

In this paper, we investigate the expression of SMAD mRNA and protein in cultured human fibroblasts of keloids, control skin and normal scars.

Our data reveal no significant difference in expression levels of SMAD2 and SMAD4 mRNA between keloids, normal scars and control skin. SMAD3 mRNA is significantly down-regulated in keloid and normal scar fibroblasts compared to control skin (Table 3). Interestingly, the ratio of SMAD2 and SMAD3 mRNA expression shows a significant down-regulation in keloid fibroblasts compared to normal scars and a significant up-regulation in normal scar fibroblasts compared to control skin. Further, we determined the mRNA expression of the inhibitory SMAD6 and 7. Keloid derived fibroblasts exhibit significantly decreased levels of SMAD6 and SMAD7 mRNA compared to normal scar derived fibroblasts. We do not find SMAD7 mRNA differences between keloids and normal skin. The effect of TGF-β1 stimulation on SMAD2, 3, 4, 6 and 7 mRNA expression in keloid, normal scar and control skin derived fibroblasts are investigated. Our results do not show a significant effect on the mRNA expression of SMAD2 and SMAD4 in the analyzed cell lines. In control skin derived fibroblasts TGF-β1 stimulation induce a significant down-regulation of SMAD3. Fibroblasts from normal scar tissue respond with a significant down-regulation of SMAD6 and 7 whereas fibroblasts from control skin show a significant up-regulation of SMAD6 and 7 after stimulation. Notably, keloid fibroblasts do not respond to TGF-β1 stimulation (Table 3).

To study the expression of SMAD proteins lysates of cultured fibroblasts from keloids, normal scars and control skin are analyzed by western blotting. In addition, the effect of TGF-β1 stimulation on SMAD protein levels is investigated. Our results do not show a significant difference of SMAD3 protein levels in the studied cell lines. TGF-β1 stimulation induce down-regulation of SMAD3 protein expression in keloids, normal scars and control skin. In contrast, TGF-β1 stimulation leads to SMAD4 protein down-regulation in normal scar and normal skin derived fibroblasts while TGF-β1 up-regulates SMAD4 protein expression in keloid derived fibroblasts. TGF-β1 stimulation induces up-regulation of SMAD7 protein in fibroblasts from normal scars and control skin whereas keloid derived fibroblasts do not show a response to stimulation (Table 3).

	Keloid	+TGF-β1	Normal Scar	+TGF-β1	Control Skin	+ TGF-β1
mRNA						
SMAD3	↓	↔	↓	↔	↑	↓
SMAD6	↓	↔	↑	↓	↑	↑
SMAD7	↓	↔	↑	↓	↓	↑
Protein						
SMAD3	↔	↓	↔	↓	↔	↓
SMAD4	↔	↑	↔	↓	↔	↓
SMAD7	↓	↔	↑	↑	↑	↑

Table 3. Relative SMAD mRNA and protein expression before and after TGF-β1 stimulation.

PAPER III

In this study, we develop a questionnaire to describe the quality of life of patients with keloids and hypertrophic scars and we test the reliability of this instrument. First, we created an item pool by screening the items of the questionnaire on Experience with Skin Complaints. 50 items are selected with regard to their relevance for patients with scars. These items are semantically modified and presented to 10 outpatients in a preliminary questionnaire. This pilot study results in a sufficient comprehensibility and acceptability of these items.

The study include 100 outpatients (66 women) with keloids and hypertrophic scars. The mean age of the study population is 36 years (range: 10-80 years, SD = 17.2). 90% of the distributed questionnaires are received completely answered after 14 days. Duration of disease is shown in table 4. The highest incidence of pathological scarring, 75% of all patients, is on the ventral side of the trunk (Figure 15). 16 items are excluded from the questionnaire as more than 50% of the patients answered to these items with “totally inaccurate”. The results of the factor analysis of the remaining 34 items lead to a two-factor solution. Factor 1, the psychological impairment, consists of nine items which describe feelings of worthlessness, the experience with a lack of physical attractiveness or sexual desirability in the context of the scars and special ways of avoiding public situations. Factor 2, the physical impairment, consists of five items characterized by physical aspects like ‘‘Changes in the weather seriously affect my scars (pain, feeling of tension)’’ or ‘‘My scars restrict my mobility’’; this factor also deals with itching and scratching in the context of scars.

The two scales psychological and physical impairment show a high test-retest reliability and both scales are independent of each other. Single items which clinically characterize the disease show correlations to scale 1 and 2. Scale 1 shows a high correlation to ‘‘Suffering from disease’’ and scale 2 shows a high correlation to ‘‘Restriction of mobility due to disease’’. There is no correlation to age, gender, and duration of disease except a moderate correlation of scale 2 and age. Visible keloids or hypertrophic scars on the head, lower arms, or lower legs have a strong influence on scale 1 ‘‘psychological impairment.’’

	Min	Max	Median	Mean	SD
Duration (years)	0.4	33.4	5.3	7.2	5.8

Table 4. Duration of disease.

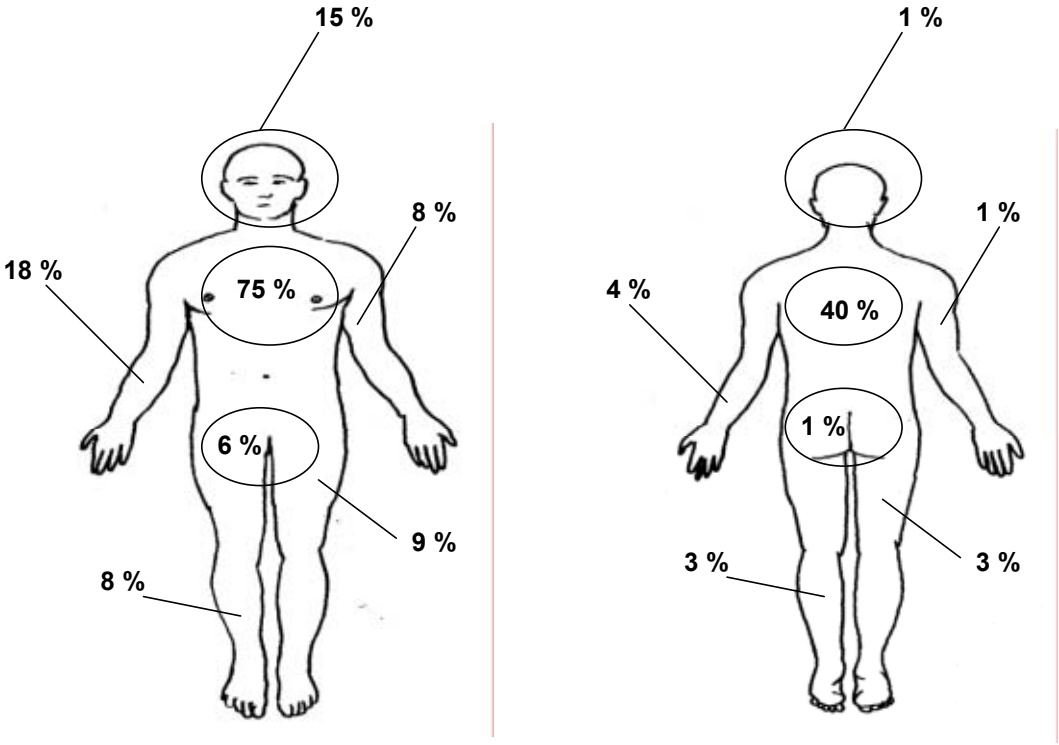

Figure 15. Localization and percentage of keloids and hypertrophic scars in the study population. Several patients have more than one scar, therefore the percentage does not add to 100%.

PAPER IV

In this paper, we investigate gene expression profiles of keloid fibroblasts from different anatomical sites of keloids using microarrays covering the whole human genome. Punch biopsies are taken from the following regions of the excised keloid tissue: From the deeper part of the keloid centre (A), the superficial part of the keloid centre (B) and from the superficial part of the active erythematous keloid margin (C) (Figure 10). Expression profiles obtained from keloid derived fibroblasts are compared to control skin fibroblasts from three volunteers.

Table 5 shows the number of regulated genes in different keloid sites compared to control skin. Most genes are found to be up– or down-regulated in the active keloid margin (C) compared to control skin.

Keloid sites compared with CS	up-regulated (>2-fold)	down-regulated (>2-fold)
A vs CS	578	208
B vs CS	599	223
C vs CS	632	243

Table 5. Number of genes more than 2-fold up- or down-regulated in different keloid sites (A, B and C) compared to control skin.

Our study reveals 105 differentially regulated genes comparing different parts of the keloids (Table 6). Comparison of the deeper central part of keloids (A) with the active margin (C) reveals most up- and down-regulated genes.

Keloid sites	up-regulated (>2-fold)	down-regulated (>2-fold)
A vs B	23	7
A vs C	41	6
B vs C	15	13

Table 6. Number of genes more than 2-fold up- or down-regulated compared in between different keloid sites (A, B and C).

An increase in extracellular matrix production and reduced degradation is one of the hallmarks in keloid development. Our study reveals a number of more than 2-fold up- or down-regulated genes involved in these processes: We find that fibroblast activation protein alpha (FAP) is up-regulated in site A, B and C compared to control skin. Parathyroid hormone-related protein (PTHrP) plays a role in cancer invasion by increasing cell proliferation. The gene coding for PTHrP is increased in all biopsy sites (A-C) compared to control skin. Calumenin (CALU), involved in processes leading to fibrosis, is more than 4-fold up-regulated in site C compared to control skin. The extracellular matrix degrading enzymes MMP3 and MMP19 show expression as followed: Matrix metalloproteinase 3 (MMP3) is decreased in site A and C compared to control skin while the matrix metalloproteinase 19 (MMP19) is more than 4-fold up-regulated in site B compared to control skin. Insulin-like growth factor binding protein 5 (IGFBP5) is involved in the regulation of wound healing. Compared to control skin IGFBP5 is up-regulated in all parts of the keloids but is down-regulated in keloid site B compared to C.

Even if their role in keloid development is unclear, we can not exclude a possible role of hyaluronan synthase (HAS1) and leukemia inhibitory factor (LIF) in the pathogenesis of keloids. HAS1 is up-regulated in all keloid parts compared to control skin and HAS1 is even up-regulated in site A compared to B and C. LIF is increased in the keloid site A compared to B, C and control skin.

The development of keloids might be due to decreased apoptosis of fibroblasts. Notably, our study shows up- and down regulation of apoptosis associated genes in keloids: The gene coding for the apoptosis caspase activation inhibitor (AVEN) is up-regulated in the keloid site C compared to control skin. Another gene, coding for a disintegrin and metalloproteinase (ADAM12) is up-regulated in site B compared to control skin. Annexin A1 (ANXA1) is up-regulated in the keloid sites A and B compared to control skin.

Besides an unbalanced ECM production and dysregulation of apoptosis, increased angiogenesis may be involved in keloid development. Our data reveal down-regulation of genes which are involved in inhibition of angiogenesis. The gene coding for pentraxin 3 (PTX3) is down-regulated in all three keloid sites compared to control skin. In addition, thrombospondin 2 (THBS2) is down-regulated in C compared to control skin. PTX3 is found to be up-regulated in keloid site A compared to site B and THBS2 is up-regulated in site A compared to keloid site C.

RT-PCR data comparing keloid site A, B, C and control skin show significantly increased expression of HAS1 and IGFBP5 in all parts of the keloid compared to control skin. LIF mRNA expression is significantly down-regulated in B compared to control skin and up-regulated in A and C. The expression of MMP3 mRNA is significantly down-regulated in C compared to control skin. PTX3 and THBS2 mRNA expression is significantly down-regulated in B and C compared to control skin. STAT1 is significantly up-regulated ($p<0.001$) in A compared to control skin.

ELISA is performed to determine the protein expression of INHBA, MCP-1, STAT1 and MMP3. INHBA is significantly up-regulated in control skin and B compared to C. STAT1 protein expression shows a significantly up-regulation in A compared to control skin. MCP-1 is significantly up-regulated in all three keloid compartments compared to control skin. Protein expression of MMP3 shows a significantly down-regulation in A and C compared to control skin.

DISCUSSION

TGF-β and TGF-β receptor expression in keloids and hypertrophic scars

An altered regulation and production of TGF-β is linked to several fibrotic diseases of the kidney, liver, lung and skin. TGF-β, its receptors and intracellular signaling molecules are also described in the pathogenesis of cancer (132). TGF-β is generally considered to be a key player in keloid development although only a few reliable studies describe the expression of TGF-β in keloids. Lee et al. find increased TGF-β1 and TGF-β2 protein expression in keloid derived fibroblasts compared to normal human skin fibroblasts whereas TGF-β3 expression did not differ (71). An increased expression of TGF-β1 mRNA in cultured keloid fibroblasts was recently reported by Fujiwara et al. (133). Further, an increased expression of TβRI and TβRII protein is described in keloid fibroblasts relative to normal human skin fibroblasts (134). In human post-burn hypertrophic scar tissues expression of TGF-β1 mRNA and protein is described to be increased (135,136) and by using immunohistochemistry, Schmid et al. find elevated expression levels of TβRI and TβRII in human hypertrophic scars (137).

Altered relation of TGF-β subtypes and receptors involved in keloid pathogenesis

Several studies describe the role of TGF-β1 in keloids and hypertrophic scars. TGF-β1 stimulation induces collagen synthesis in keloid and hypertrophic scar fibroblasts (70,103) and anti-TGF-β1 antibody is capable of reducing the rate of collagen synthesis of keloid fibroblasts (103). Our data reveal increased expression of TGF-β1 mRNA in keloid derived fibroblasts confirming results from other studies (71,133). Whether autocrine TGF-β1 production stimulates keloid fibroblasts directly or via endothelial cells as suggested by Peltonen et al. (9) remains unclear. Synergistic effects and complex paracrine interactions are most likely as even keloid derived keratinocytes produce increased levels of TGF-β1 (138). Interestingly, TGF-β1 up-regulation alone seems not to be sufficient for excessive scarring. Gene-modified fibroblasts over-expressing either the active TGF-β1 or the latent molecule are implanted in the dermis of athymic mice. Only fibroblasts expressing active TGF-β1 form "keloid-like" nodules implying that other signals are needed to activate the latent TGF-β1 (139).

In animal models TGF-β2 has been suggested to induce cell proliferation and collagen production in scars (140,141). However, comparisons between keloid pathogenesis and results from experimental models of scarring in animals has to be made with caution in particular as animals do not have the ability to develop keloids. Our data from human skin reveal similar level of TGF-β2 mRNA expression in keloids and control skin and an increased expression in hypertrophic scars. Lee et al. describe increased TGF-β2 protein expression in keloid derived fibroblasts compared to normal skin fibroblasts. This discrepancy in results is hard to explain as Lee et al. did not define the biopsy site from which the fibroblasts are harvested and the time points for analysis of mRNA and protein expression may diverge.

The differential diagnosis between keloids and hypertrophic scars is sometimes difficult and may also be a confounder. This underlines the importance of standardized conditions concerning clinical diagnosis, biopsy site, and cell culture. Based on our findings we propose that TGF-β2 alone does not induce fibrosis, instead, a certain combination of TGF-β subtypes and their receptors control ECM synthesis.

The role of TGF-β3 in wound healing and pathological scarring is not fully understood, but TGF-β3 is both, scar inducing and scar reducing in animals (142,143). Murata et al. show that TGF-β3 stimulation increase DNA and collagen synthesis and α1(I) procollagen mRNA levels in human skin fibroblasts. In contrast with these stimulatory effects they show a down-regulation of α1(I) and α1(III) procollagen and TGF-β1 mRNA levels when TGF-β3 is added in combination with TGF-β1 and conclude that stimulation of collagen synthesis by TGF-β3 occurs both through a TGF-β1-dependent and independent pathway. By downregulating the response to TGF-β1 and by shifting from one pathway to the other, TGF-β3 may regulate collagen deposition (144). Our data from keloid fibroblasts show a significantly decreased expression of TGF-β3 mRNA in keloids compared to hypertrophic scars and to control skin. If the results from Murata et al. hold true TGF-β3 downregulates the response of TGF-β1 and reduce the overall TGF-β induced program of collagen deposition. Consequently, our results of a reduced TGF-β3 expression suggest a possible role of TGF-β3 in the formation of keloids. The high expression of TGF-β3 in hypertrophic scars might reflect controlled fibrosis and may hypothetically explain why hypertrophic scars do not grow invasively into the surrounding healthy skin. PAI-1, which inhibits the activation of plasmin and thus leading to diminished fibrin degradation, is a marker of fibrosis. PAI-1 is highly expressed in the epidermis and dermis of TGF-β3 knockout mice (145). These findings support our hypothesis that the absence of TGF-β3 plays an important role in keloid formation.

Schmid et al. (137) demonstrate elevated expression levels of TβRI and TβRII in hypertrophic scars. They interpret this as a failure to eliminate TβR-overexpressing fibroblasts during granulation tissue remodeling leading to a persistent autocrine, positive-feedback loop resulting in overproduction of ECM. Similarly, Kawakami et al. show increased TβRI and TβRII mRNA levels in scleroderma fibroblasts (146) and suggest that elevated production of type I collagen by scleroderma fibroblasts results from overexpression of TβRs. Denton et al. show that expression of a non-signaling, kinase-deficient human type II TGF-β receptor (TbetaRIIDeltak) in fibroblasts of transgenic mice induce dermal and pulmonary fibrosis. Transgenic fibroblasts proliferate more rapidly, produce more ECM and show increased expression of PAI-1 and connective tissue growth factor (CTGF). These findings support a role of TβRII in fibrosis (147). Interestingly, our data reveal a significant down-regulation of TβRII in keloids compared to hypertrophic scars. In the light of Dentons study, we suggest that diminished TβRII expression provoke proliferation of fibroblasts and ECM production in keloids. Geiser et al. and Goldberg et al. suggest that TβRII is necessary for mediating the effects of TGF-β on inhibition of growth (148,149). Our results strengthen the idea that reduced TβRII expression is involved in keloid pathogenesis.

The important role of TβRII in inhibiting TGF-β1 mediated ECM production and cell proliferation is further supported by the findings of McCaffrey et al (150). They show that transfection and overexpression of TβRII in smooth muscle cells derived from atherosclerotic lesions inhibits TGF-β1 mediated cellproliferation and ECM production. In this perspective our findings of a significant reduced TβRII mRNA expression, increased ratio of TβRI/TβRII mRNA and an increased level of TGF-β1 mRNA may in a similar manner explain the fibroproliferative phenotype of keloids.

Contrary to keloids our findings in hypertrophic scars show an increased TβRII expression and a decreased TβRI/TβRII ratio. As hypertrophic scars do not grow invasively into the surrounding healthy skin, but cease growing after a period of time, the increased TβRII expression and decreased TβRI/TβRII ratio might reflect the inhibition of ECM production. In order to clarify this point the TβRI/TβRII ratio has to be studied in more detail in hypertrophic scars over time.

Our results demonstrate a combination of increased TGF-β1 and 2, decreased TGF-β3, and increased TβRI/TβRII ratio in keloid fibroblasts. This phenotype may be involved in the development of keloids. The proportion between TGF-β subtype and receptor expression possibly will determine whether fibroblasts have a balanced ECM production or not. As shown in Figure 14, control skin fibroblasts exhibit higher levels of TβRII and TGF-β3 together with reduced levels of TGF-β1 compared to keloid fibroblasts. The factors contributing to the different phenotypes seen in keloid and control skin fibroblasts are still unknown and further studies are needed to identify those factors.

SMAD signaling in keloids

The TGF-β signal is mediated by intracellular proteins, called SMADs. Changes in this signaling pathway regulate TGF-β expression and induce gene expression in the cell. The SMAD signaling pathway is crucial for simultaneous activation of several collagen genes by TGF-β and about 60 other ECM-related genes are identified as gene targets downstream of TGF-β (151).

The expression of SMAD proteins in normal scars is not analyzed and only few studies describe the expression in keloids and hypertrophic scars. Phan et al find increased basal levels of SMAD2, 3 and 4 protein in keloid fibroblasts. When these fibroblasts are co-cultured with keloid derived keratinocytes there is an up-regulation of SMAD3 and phosphorylated SMAD2 protein as well as an enhanced SMAD3 phosphorylation and SMAD2/3/4 binding complex production (152).

Tsujita-Kyutoku et al. show up-regulation of SMAD2 and 3 protein expression in fibroblasts derived from central parts of keloids (153).

Inhibition of SMAD3 in keloid fibroblasts leads to reduced expression of CTGF (154) and silencing SMAD2 by siRNA induce decreased procollagen expression (155) suggesting a functional role of SMAD proteins in keloid development. Interestingly, a linkage analysis in a Chinese pedigree suggests SMAD to be a possible gene involved in keloid pathogenesis and a susceptibility locus on chromosome 18q21.1 (156).

Disturbed SMAD signaling pathway and decreased expression of inhibitory SMAD proteins in keloids

Our study show significantly reduced SMAD3 mRNA expression in keloids and in normal scar fibroblasts compared to control skin. No difference is seen in keloids compared to normal scar fibroblasts. SMAD2 and SMAD3 may act as antagonists and Labbe et al. suggest their relative levels to correlate to the biological response (157). Therefore, we investigated the ratio of SMAD2/SMAD3 mRNA. This ratio is significantly decreased in keloids and in control skin compared to normal scars. The decreased SMAD3 and decreased SMAD2/SMAD3 expression in keloids might lead to enhanced matrix synthesis in keloids. In contrast, the decreased SMAD3 but increased SMAD2/SMAD3 expression in normal scars might prevent over-expression of ECM in normal scars.

In line with a previous report, we find a down-regulation of SMAD3 mRNA after TGF-β1 stimulation in control skin fibroblasts (158), but we find no modulatory effects of TGF-β1 stimulation on SMAD3 mRNA in keloids and normal scars, reflecting differences in TGF-β1 signal transduction pathways between keloid, normal scar and control skin fibroblasts. One recent study suggest SMAD-independent pathways leading to accelerated and pathologic scarring (159), but further studies are needed to elucidate possible alternative SMAD pathways playing a role in keloid scarring.

As opposed to the finding at the mRNA level we detect no significant difference of SMAD3 protein studying fibroblasts from keloids, normal scars and control skin. However, following TGF-β1 stimulation SMAD3 protein is down-regulated in keloids, normal scars and control skin. The incongruent results between mRNA and protein expression might be due to mRNA stability or due to the time point analyzing protein expression (160).

SMAD signaling plays an important role in developmental and cancer biology as well as in fibropathogenesis. The use of mice with a targeted deletion of SMAD3 shows that most of the pro-fibrotic activities of TGF-β are mediated by SMAD3. The loss of SMAD3 interferes with TGF-β-mediated induction of genes for collagen, PAI-1 and TIMP-1 (161). SMAD3 null mice are resistant to radiation-induced cutaneous fibrosis and bleomycin-induced pulmonary fibrosis (162). There is no animal model for keloids but several experimental animal models of fibrotic diseases like scleroderma, cystic fibrosis and cirrhosis implicate involvement of SMAD3 and increased SMAD3 mRNA and protein levels are found in fibroblasts of patients with scleroderma (163). Further, the inhibition of SMAD3 by overexpression of the inhibitory SMAD7 reduces the fibrotic response in animal models of kidney, lung, liver and radiation-induced fibrosis (164). In contrast to these results decreased SMAD3 protein expression leads to increased and accelerated wound healing in mice (165). It is not possible to directly translate results from the above mentioned animal studies to the human skin and keloids.

In our study the basal expression of SMAD4 mRNA as well as SMAD4 protein is the same in keloids, normal scars and control skin, but TGF-β1 stimulation induces up-regulation of SMAD4 protein in keloids and down-regulation in normal scars and control skin fibroblasts. On the contrary, Phan et al. show high basal levels of SMAD4 protein in keloid fibroblasts compared to control skin fibroblasts (152).

The deviating results may be explained by the variation in inclusion criteria, duration of disease, and biopsy site within the keloids. SMAD4 participates in regulating the transcription of ECM coding genes. Increased levels of SMAD4 in keloid fibroblasts after TGF-β1 stimulation may lead to increased signal transduction and to increased fibrosis. Our data point towards a disturbed SMAD4 signaling in keloid fibroblasts. Notably, in scleroderma fibroblasts SMAD4 protein and mRNA levels are not elevated (163). These results are in line with our findings in keloids.

In the present study we demonstrate for the first time decreased SMAD6 mRNA in keloids compared to normal scar and control skin. SMAD7 mRNA is significantly reduced in keloids as well as control skin compared to normal scars and its protein is less expressed in keloids compared to normal scars and control skin. High expression of the inhibitory SMAD6 and 7 inhibits TGF-β signaling leading to controlled proliferation and collagen deposition in normal scars. The decreased expression of inhibitory SMADs in keloids may cause a persisting TGF-β signal. Interestingly recent studies show that decreased SMAD7 contributes to cardiac fibrosis (166), to TGF-β hyper-responsiveness in scleroderma (167,168), and to the development of pulmonary fibrosis (169). Further, SMAD7 inhibits the expression of ECM genes in hypertrophic scar fibroblasts (170). We hypothesize that SMAD7 may exert beneficial effects on excessive scar formation and is an interesting target for future treatment strategies.

To summarize, we find a decreased SMAD2/SMAD3 mRNA ratio in keloids and diminished SMAD3 mRNA. Only keloid fibroblasts revealed up-regulation of SMAD4 protein after TGF-β stimulation and the expression of the inhibitory SMAD6 and 7 are reduced in keloids.

Future studies are required to analyze the role of phosphorylated SMAD proteins (p-SMAD) in keloid development as p-SMADs are the biological active proteins. It is of interest to study the translocation of p-SMADs in the cell nucleus to verify whether or not gene transcription is induced. Further, our present results from *in vitro* studies have to be verified *in vivo*. In the future, the interaction of TGF-β/SMAD-signaling with Smurf, an ubiquitin ligase, and SMAD anchor for receptor activation (SARA) has to be analyzed. Smurf is involved in degradation of R-SMADs and degrades SMAD/ TβR complexes (171). The recognition of R-SMADs by TβRs is facilitated by auxiliary proteins. SMAD2 and 3 are immobilized near the cell surface by SARA (172). SARA allows more efficient recruitment of SMAD2 and 3 to the receptor for phosphorylation. Finally, it would be very interesting to study the role TGF-β/ TβR-complex processing in keloid pathogenesis. The activated TGF-β/ TβR-complex undergoes endocytosis via two distinct routes: via coated vesicles to early endosomes for signaling and via caveolae to caveolin-positive vesicles for degradation (173).

Decreased quality of life of patients with keloids

Moderate and common inflammatory skin diseases have been shown to impair QOL to the same extend as life threatening diseases (115). Physical as well as psychological problems have been described in patients with burn scars (174,175). Some QOL instruments for skin diseases have been developed as for example the DSQL (109) or the PSORIQoL (176), a psoriasis-specific instrument.

No validated questionnaire for investigation of the impairment of the quality of life in patients with keloids has been published. Therefore, we developed a new questionnaire to investigate and measure the quality of life of patients with keloids and hypertrophic scarring. Although other QOL instruments exist they do not include the factors known to be associated with pathological scarring and which may not respond to medical treatment. The number of hypertrophic scars and keloids is increasing as decorative piercing is more often used particularly in younger age groups and in visible anatomical sites (177). Keloids and hypertrophic scars can restrict mobility when present over joints. Many patients report itch, pain, and discomfort. Treatment of keloids and hypertrophic scars is difficult, often painful, longlasting, and unsatisfactory (178). Our impression from meeting patients suffering from pathological scarring is that they are constantly aware of their skin symptoms and the psychological consequences. Therefore, it can be assumed that the quality of life of patients with keloids and hypertrophic scars may be severely impaired.

Our study population is relatively young (mean age 36 years) correlating with the typical age for keloid patients (179). We assume that this young age group has a higher tendency to seek treatment for their scars. Shaffer et al. describe that the prevalence of keloids is equal in male and female patients (178). Our data reveal fewer male patients in the study (34%) and we hypothesize that female patients are more prone to seek treatment for their scars, especially when they are visible. The mean duration of disease is found to be seven years. The long duration and treatment failures might have a profound impact on the quality of life. 75% of the scars are localized in the presternal area, an area exposed in women. Our results are according to the literature as this area is most commonly affected by keloids (179).

Our results show that quality of life in patients with keloids and hypertrophic scarring can be described by two scales: ‘‘psychological impairment’’ and ‘‘physical impairment.’’ Scale 1 reflect the psychological impairment of the patients (e.g. item 7 ‘‘I do my best to prevent even people close to me from knowing that I have scars’’ and item 11 ‘‘I don’t visit the swimming pool or the sauna since other people could feel disgusted because of my scars’’). These items clearly describe the decreased quality of life of these patients. They recognize rejection, loss of self-confidence and they try to hide their scars. Scale 2 reflect the physical impairment (e.g. item 2: ‘‘My scars restrict my mobility’’ and item 6 ‘‘I find it difficult to put up with the itching caused by my scars’’). We find discriminating psychometric values of both our scales. First, they are relatively independent from each other, and second, both the timeless internal consistency (Chronbach’s alpha) and the time-dependent retest-reliability are sufficient. This study show that our new questionnaire is largely independent of specific social or demographic parameters. There is a slight relationship between scale 2 and age. The high relation to items of clinical symptoms suggests sufficient external validity of the scales.

The results of this study demonstrate for the first time a severe impairment of quality of life of patients suffering from keloids and hypertrophic scars. The results suggest that the questionnaire enables to measure the quality of life in these patients. Visible scars on the head, lower arms, or lower legs significantly influence scale 1 “psychological impairment” implying the role of visible scars as an important stigma.

This questionnaire may be useful to document the impact of new developments in the treatment of pathological scarring on quality of life. Future studies in this field should include patients with normal scarring to further support the validity of the scales shown in this study.

Altered gene expression patterns in different keloid parts

There are two previous studies in which gene expression profiles in keloids have been analyzed with microarray technique. In the most recent study 22000 genes are analyzed in keloid fibroblasts compared with normal skin fibroblasts and 43 genes are up- and 6 down-regulated (180). In this study there is a wide age range of subjects and the site of the biopsy within the keloid is not presented. In addition, the race of the individuals and the reason for keloid development is not recorded. Chen et al. performed microarray analysis of three keloids and three normal skin samples in Chinese patients (181). The keloids developed after burn injury. In this study 250 genes are up- and 152 genes down-regulated.

Our study is the first comparing gene expression profiles between different lesional sites of keloids. The microarray chip used in our study covers approximately 47000 transcripts (38500 genes). This may explain the low number of regulated genes in other studies compared to our results. All keloids investigated in our study developed spontaneously and show clinical signs of regression in the centre (see Figure 7) and are actively growing into the surrounding healthy skin at the margin. The results of our study support the important role of the biopsy site for studies in keloids as our results show that different genes are regulated in different sites of keloids.

Despite the enormous potential of the microarray technique, several issues needs a careful approach using this instrument. First, the disease and disease activity have to be defined. Second, heterogeneous cell populations in the tumour sample may confound the results. It can not be excluded that our results are influenced by the gene expression profiles of subpopulations of keloid fibroblasts and that this profile changes under *in vitro* conditions. Third, there is a lack of standardisation of microarray chips in the numbers, nature and volume of probes (spots), reproducibility of results and different approaches to data analysis. This makes comparison between published microarray data difficult.

The clinical regression of keloids, often seen in the centre, may be due to different biological processes as increased frequency of apoptosis, ECM degradation or remodeling. Interestingly, we find several apoptosis inducing genes up-regulated in fibroblasts from the central regressing part (B) of the keloids. One is ADAM12, which has been shown to increase the apoptotic sensitivity of non-neoplastic cells (182). CCAR1 and ANXA1, known to be pro-apoptotic genes, are both up-regulated in the keloid regression site (B). (183,184). We propose that these apoptosis related genes are essential for clinical regression of keloids.

We find a localized overexpression of MMP19, known to be involved in ECM degradation, in the regression part of keloids (B) (185).

MMP3, involved in wound healing and tissue remodeling, is a key enzyme in degrading ECM, fibronectin, laminin, and collagen III, IV, IX, and X (186). Our data show down-regulation of MMP3 in all three keloid compartments (Figure 10; A, B and C) compared to control skin leading to decreased ECM degradation in keloids. Interestingly, our data reveal a spatially relative up-regulation of MMP3 in the regressing part of the keloid (B) compared to the other keloid compartments leading to increased ECM breakdown.

Other genes of interest in keloid development are HAS1 and LIF. Up-regulation of HAS genes by bFGF and IGF-1 is closely associated with the stimulation of hyaluronan acid (HA) synthesis. HA is actively produced during wound healing and tissue repair to provide a framework for redeveloping blood vessels and fibroblasts. Effects of growth factors like bFGF and IGF-1 on HAS gene expression may have important implications for tissue remodeling (187). The expression of HAS1 in keloids has not been described before. Our data reveal a localized up-regulation of HAS1 in the deeper part of the keloid centre (A) which might be a sign of a shift in phenotype of keloids from an uncontrolled ECM production to a more normal wound healing process, but the role of HAS1 in keloid development can at this point only be speculated. LIF is active in a number of biological processes as inflammation, proliferation and differentiation. Enhanced LIF expression is found in various cutaneous neoplasms leading to increased angiogenesis and matrix production (188). We demonstrate for the first time a low expression of LIF in the regression part of the keloids compared to the other keloid compartments (A and C) as well as compared to control skin indicating a pathogenetic role of LIF in keloid development.

In contrast to the regression site in the centre the active margin of keloids is aggressively growing. Increased ECM production, angiogenesis, and anti-apoptotic processes contribute to the invasive growth of keloids at the active margin. Notably, we discover several genes implicated in ECM-production up-regulated at the active margin of keloids (C). FAP belongs to the serine protease family and is expressed in reactive stromal fibroblasts of epithelial cancers and in granulation tissue of healing wounds. The localization of FAP at the cell surface has a role in processing soluble factors and in degrading ECM components. This has been shown to be essential in tumour invasion, angiogenesis and metastasis (189). Our data demonstrate an increased expression of FAP in all compartments of the keloid compared to control skin. FAP might have dual actions as high expression of FAP enables keloid fibroblasts to invasive growth into the surrounding healthy skin and FAP also converts α2 antiplasmin into a more active form leading to reduced ECM degradation and increased scar formation (190).

PTHrP has been suggested to play a role in cancer invasion by increasing cell proliferation and adhesion to the ECM via upregulation of proinvasive integrin expression (191). Interestingly, in our study keloid fibroblasts at the active margin (C) show the most prominent expression of PTHrP leading us to suggest that PTHrP plays a role for the invasive character of keloids. We find the calcium-binding protein CALU up-regulated in the active margin of keloids (C). As CALU has been reported to be up-regulated in lung fibrosis (192) it is possible that CALU is involved also in the development of dermal fibrosis.

Mukhopadhyay et al. show increased INHBA mRNA expression in keloid fibroblasts and increased localization of INHBA protein in the basal layer of epidermis of keloid tissue compared to control skin. The authors suggest that INHBA is a key regulator in keloids as INHBA up-regulates plasminogen activator inhibitor-1 (PAI-1) which in turn inhibits ECM and collagen degradation in keloid fibroblasts (193). In line with these findings our study reveal increased mRNA expression in all keloid compartments compared to control skin. Notably, we find a down-regulation of INHBA on protein level at the active margin of keloids (C) compared to control skin. A distinct diminished INHBA protein level in fibroblasts at the active keloid margin support the invasive character of these fibroblasts as low levels of INHBA inhibit PAI-1 and in turn augment ECM degradation.

Another interesting aspect in keloid development is the expression of MCP-1. There is recent evidence suggesting that MCP-1 participate in fibrotic processes by inducing the secretion of ECM components (194,195). This gene has not been studied in keloids but our data reveal an up-regulation of MCP-1 protein in all parts of the keloid compared to control skin. In this light MCP-1 might play an important role in the complex regulatory network of keloid formation.

IGF-I is one of the most important growth factors in wound healing stimulating keratinocyte and fibroblast proliferation as well as ECM synthesis. Co-stimulation of IGF-I and TGF-β1 markedly increase production of extracellularmatrix proteins in keloid fibroblasts compared to control skin. IGF-I treatment alone has no stimulatory effect (196-198). Our data show no difference in IGF-I mRNA in keloids compared to control skin. In wounds IGF-I is complexed to IGFBPs and unavailable for receptor interaction. IGFBPs undergo limited proteolysis by PAPPA to deliver bioactive IGF-I and stimulate new growth at the wound site (199). It might be worth mentioning that our data reveal up-regulation of IGFBP5 and 7 in all parts of the keloid compared to control skin. The inhibitory effect of IGFBP is reduced by up-regulation of PAPPA. This system and his actions in keloid development needs further analysis of the levels of IGF-1, IGFBP, and PAPPA in order to understand their role in keloid pathogenesis.

Keloids present themselves as erythematous tumours in the active margin and angiogenesis appears to play an important role. Therefore genes involved in pathways regulating angiogenesis are of importance for the understanding of keloid pathogenesis. PTX3 is a secreted molecule similar to classical pentraxins (e.g. C-reactive protein) and plays a role in wound healing (200) by binding to FGF-2 which leads to inhibition of angiogenesis (201). In this study we find decreased PTX3 mRNA in all keloid compartments compared to control skin which might lead to increased angiogenesis. THBS2 gene is mediating cell-to-cell and cell-to-matrix interactions and has been shown to functioning as a potent inhibitor of tumour growth and angiogenesis (202). THBS2 has not been described before as a possible pathogenic factor for keloid development but our results reveal a decreased expression of THBS2 in keloids, which might have some impact on angiogenesis and growth.

Regulated cell death (apoptosis) is one of the most regulated biological processes. The balance between pro- and anti-apoptotic factors determines tissue homeostasis. AVEN represents a new class of cell death regulators suppressing apoptosis induced by Apaf-1 and caspase-9 (203,204).

Our study shows a localized overexpression of the novel apoptosis inhibitor AVEN at the active margin of keloids leading to fibroplasia and increased ECM-production.

Our results point towards a highly complex interaction of genes involved in the pathophysiology of keloids. We determine several novel genes previously not described to be regulated in keloids. For the first time we make the important distinction of unique gene expression profiles in different lesional sites of keloids reflecting different stages in the live cycle of keloids. We suggest that activation of pro-apoptotic genes as ADAM12 or inhibiting ECM inducing genes as INHBA or MCP-1 might be possible genes for new future treatment strategies for keloids.

CONCLUSIONS

General conclusions

In this thesis we describe an altered expression pattern of TGF-β subtypes and their receptors in keloid derived fibroblasts. These findings suggest a certain combination of TGF-β and its receptors accountable for the excessive ECM production in keloids. SMAD proteins play an important role in TGF-signaling and our results implicate disturbances in the SMAD signaling pathway in keloid pathogenesis. Furthermore, fibroblasts obtained from different keloid parts exhibit an unique gene expression pattern. These specific gene expression patterns may explain the different growth activity in the studied keloid sites. Finally, we develop a new instrument to determine the quality of life in patients with keloid scars, demonstrating an impairment of quality of life even when a limited area of the skin is involved.

Specific conclusions and future perspectives

- ❒ mRNA expression of TGF-β1 and TGF-β2 is increased in keloid derived fibroblasts. This may be involved in up-regulated ECM production. We find decreased TGF-β3 mRNA which is an additional factor signaling ECM overproduction. Future studies are needed to elucidate the role of specific TGF-β expression patterns in keloid development.

- ❒ The expression of TβRII mRNA is diminished in keloid fibroblasts and the ratio of TβRI/TβRII mRNA is increased which may contribute to keloid pathogenesis. It is unclear whether the receptor ratio alone or in combination with altered TGF-β expression is inducing keloids. Further studies may help to answer this question.

- ❒ Our data reveal decreased SMAD3 mRNA expression and decreased ratio of SMAD2/SMAD3 mRNA implicating a disturbed SMAD signaling pathway in keloid fibroblasts.

- ❒ We find keloid fibroblasts to up-regulate SMAD4 protein after stimulation with TGF-β1 resulting in diminished levels of the inhibitory SMAD proteins SMAD6 and 7 in keloid fibroblasts. This may contribute to unlimited and de-regulated TGF-β signaling and accordingly to an enhanced ECM production. Inhibition of SMAD4 or activating of SMAD7 might be an approach to reach connective tissue homeostasis in keloids.

- ❒ Gene expression patterns in the central part of keloids with clinical signs of regression, involve up-regulation of apoptosis inducing genes as ADAM12 and ECM degrading genes as MMP19. These genes may contribute to regression of keloids and are possible future target genes for prevention and treatment of keloids.

- ❒ Overexpression of apoptosis inhibitors as AVEN and down-regulation of angiogenesis inhibiting genes as PTX3 at the active margin of keloids may be responsible for the invasive character of the keloid margin.

- ❒ Our disease specific instrument for keloid disease can be used to measure the quality of life of patients with keloid scars. In addition, this instrument might be a useful tool to measure improvement of keloid treatment over time.

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