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Decreased expression of inhibitory SMAD6 and SMAD7 in keloid scarring

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Summary Keloids are benign skin tumours occurring during wound healing in genetically predisposed patients. They are characterised by an abnormal deposition of extracellular matrix components, in particular collagen. There is evidence that transforming growth factor-beta (TGFβ) is involved in keloid formation. SMAD proteins play a crucial role in TGFβ signaling and in terminating the TGFβ signal by a negative feedback loop through SMAD6 and 7. It is unclear how TGFβ signalling is connected to the pathogenesis of keloids. Therefore, we investigated the expression of SMAD mRNA and proteins in keloids, in normal skin and in normal scars.

Dermal fibroblasts were obtained from punch-biopsies of keloids, normal scars and normal skin. Cells were stimulated with TGFβ1 and the expression of SMAD2, 3, 4, 6 and 7 mRNA was analysed by real time RT-PCR. Protein expression was determined by Western blot analysis.

Our data demonstrate a decreased mRNA expression of the inhibitory SMAD6 and 7 in keloid fibroblasts as compared to normal scar ($p<0.01$) and normal skin fibroblasts ($p<0.05$). SMAD3 mRNA was found to be lower in keloids ($p<0.01$) and in normal scar fibroblasts ($p<0.001$) compared to normal skin fibroblasts.

Our data showed for the first time a decreased expression of the inhibitory SMAD6 and SMAD7 in keloid fibroblasts. This could explain why TGFβ signaling is not terminated in keloids leading to overexpression of extracellularmatrix in keloids. These data support a possible role of SMAD6 and 7 in the pathogenesis of keloids.

Keloids represent a clinically distinct form of pathological scarring which occurs during wound healing in genetically predisposed individuals. They are a unique human dermal fibroproliferative

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disorder usually induced by trauma such as burning injury and surgery.

The highest incidence of keloids is found in the black population, where it has been estimated around 4–6%[1] and up to 16% in random samples of black Africans.[2,3]

The quality of life of patients with keloids is strongly impaired by symptoms like itching and pain and in addition keloids represent a major cosmetic burden to the patients.[4]

In contrast to hypertrophic scars keloids grow invasively into the surrounding healthy skin and are not confined to the border of the original wound. Recurrence is common after surgical excision.[5] Keloids are characterised by hyperproliferation of fibroblasts and increased extracellular matrix.[6] Alteration of apoptosis and cell proliferation have been implicated in the etiology of keloids.[7] However, the pathogenesis of the development of keloids remains unknown.

There is evidence that the pathogenesis of keloid formation may involve the expression of transforming growth factor-beta (TGFβ). Three mammalian TGFβ isoforms (TGFβ1, TGFβ2 and TGFβ3) with similar but not identical bioactivities have been described. TGFβ1 and TGFβ2 proteins were found to be expressed higher in keloid fibroblasts compared with normal human dermal fibroblasts.[8] Keloid-derived fibroblasts (KFs) show a reduced growth-factor requirement in vitro,[9,10] a unique sensitivity to TGFβ[6,11–14] coupled with an increased production of TGFβ1.[8] This could explain the increased proliferation and collagen production and greater proliferative capacity than hypertrophic scar derived or normal skin derived fibroblasts.[7,15]

SMAD proteins are intracellular signaling molecules that act downstream of TGFβ receptors. The term 'SMAD' is derived from a combination of homologue genes found in *Caenorrhabditis elegans*, SMA, and *Drosophila melanogaster*, MAD.[16] A total of nine vertebrate SMADs have been identified,[17–21] as well as two in Drosophila and five in nematodes.

SMAD1, 2, 3 and possible SMAD5 interact with and are phosphorylated by specific type I serine/threonine kinase receptors and thereby act in a pathway-restricted fashion.[22] They are called pathway-restricted SMADs or receptor-regulated SMADs. SMAD2 and 3 are direct substrates for the TGFβ type I receptor, a serine/threonine kinase. Once phosphorylated and activated, these SMADs form hetero-oligomeric complexes with a second class of SMADs, the common SMADs (SMAD4). Inhibitory SMADs (SMAD6 and SMAD7) antagonize the activity of the receptor-regulated SMADs leading to termination of the TGFβ signal.

Until today there is no report how TGFβ signaling might be involved in the pathogenesis of keloids. The purpose of the present study was to investigate the expression of SMAD proteins in keloid fibroblasts. We analysed the expression of SMAD 2, 3, 4, 6 and SMAD 7 at mRNA and protein level in cultured fibroblasts and in tissue samples from keloids, normal skin and normal scars.

Methods

Primary skin fibroblast cultures

Since, one problem associated with studies in keloid patients is a proper definition of keloids the selection of patients was performed by experienced dermatologists (OB, UM). Ethical approval was obtained before the start of the study. Patients gave informed consent prior to their participation. Age-, gender- and site-matched skin from normal scars, normal skin and keloids were obtained in the same way.

Primary human fibroblast cultures were prepared from fresh tissue punch-biopsies (5 mm diameter). Fibroblasts were stored in T75 flasks (Nunc, Life Technologies Ltd, Germany). Monolayer-cultures were 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 foetal calf serum (FCS) (PAA Laboratories, Linz, Austria) and 25 mmol/l *N*-2-hydroxylpiperizine-*N*′-2-ethanesulphonic acid (HEPES) (cell concepts). Cells were incubated at 37 °C in a 5% (v/v) CO_2 humidified atmosphere. Cells from passages 2–4 were used.

Stimulation of cultured skin fibroblasts with TGFβ1

Twenty-four hours before treating cultured fibroblasts with TGFβ1, the regular medium was replaced with serum free medium. After washing twice with PBS, subconfluent fibroblasts were incubated with various concentrations of TGFβ1 (R&D Systems, Wiesbaden, Germany) diluted with 1% bovine serum albumin (BSA) (PAA Laboratories) in RPMI-1640 at different time points. Cells incubated with 1% BSA RPMI-1640 medium served as negative controls. Fibroblasts were stimulated for 1, 2, 6 and 24 h with 20 ng/ml TGFβ1 for real time PCR and Western blot analysis.