

INVESTIGATIVE REPORT

Studies of Transforming Growth Factors Beta 1–3 and their Receptors I and II in Fibroblast of Keloids and Hypertrophic Scars

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Keloids are benign skin tumours occurring during wound healing in genetically predisposed patients. They are characterized by an abnormal deposition of extracellular matrix components, particularly collagen. There is uncertain evidence that transforming growth factor-beta (TGF β) is involved in keloid formation. Therefore we investigated the expression of TGF β 1, 2 and 3 and their receptors in keloids, hypertrophic scars and normal skin. Dermal fibroblasts were obtained from punch biopsies of patients with keloids and hypertrophic scars and from normal skin of healthy individuals. Total RNA was isolated and the expression of TGF β 1, 2 and 3 and of TGF β receptors I and II (TGF β RI and II) was analysed by real-time PCR using the Lightcycler technique. Our data demonstrate significantly lower TGF β 2 mRNA expression in hypertrophic scar fibroblasts as compared with fibroblasts derived from keloids and normal skin ($p < 0.05$). In contrast, TGF β 3 mRNA expression was significantly lower in keloid fibroblasts in comparison with fibroblasts derived from hypertrophic scar and normal skin ($p < 0.01$). TGF β RI mRNA expression was significantly decreased in hypertrophic scar fibroblasts ($p < 0.01$) and TGF β RII mRNA expression was decreased in keloids compared with hypertrophic scar fibroblasts ($p < 0.001$). The ratio of TGF β RI/TGF β RII expression was increased in keloids compared with hypertrophic scar and normal skin fibroblasts. As recently supposed, an increased TGF β RI/TGF β RII ratio could promote fibrosis. Therefore our data support a possible role of TGF β RI and TGF β RII in combination with a certain TGF β expression pattern as fibrosis-inducing factors in keloids. *Key words:* wound healing; fibroblasts; TGF β receptors.

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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 disorder usually induced by trauma such as burning injury and surgery or by inflammation such as cystic acne.

The highest incidence of keloids is found in the black population, where it has been estimated to about 4–6% (1) or up to 16% in random samples of black Africans (2, 3).

In contrast to hypertrophic scars (HS), keloids grow invasively into the surrounding healthy skin and are not confined to the border of the original wound. Keloids seldom show any tendency to regress spontaneously. Recurrence is common after surgical excision, which often exacerbates the condition (4).

Hyperproliferation of fibroblasts, leukocyte infiltration and prolonged high rates of collagen synthesis characterize keloids (5). Alteration of apoptosis and cell proliferation has been implicated in the aetiology (6). However, the pathogenesis of keloids remains unknown.

Three mammalian TGF β isoforms (TGF β 1, TGF β 2 and TGF β 3) with similar but not identical bioactivities have been described. TGF β 1 and TGF β 2 proteins were recently found to be more highly expressed in keloid fibroblasts than in normal human dermal fibroblast cultures (7). Furthermore, TGF β 1, 2 and TGF β RI (TGF β receptor I) were found to be increased in HS compared with normal skin (NS). In contrast, TGF β 3 and TGF β RII expression were decreased in HS compared with normal skin (8).

Keloid fibroblasts show a unique sensitivity to TGF β . Treatment with TGF β 1 results in stimulation of total protein synthesis in normal dermal fibroblasts but not in keloid fibroblasts. TGF β 1-induced increase in fibronectin biosynthesis occurs more rapidly in keloid fibroblasts and leads to overproduction of extracellular matrix components. In addition, keloid fibroblasts were found to produce up to 12 times more collagen and TGF β 1 treatment up-regulated procollagen type I carboxyterminal propeptide (P1CP) production (5, 9–12). Keloid fibroblasts show a greater proliferative capacity than HS-derived or NS-derived fibroblasts (13). However, to date, investigations performed to identify a genetic background of TGF β expression in keloid patients have not demonstrated TGF β gene polymorphisms (14, 15).

The purpose of the present study was to investigate whether there was an aberrant expression of TGF β 1, 2 and 3 or of the two surface receptors for TGF β (TGF β RI and II). Fibroblast cultures were established

from freshly taken skin biopsies and further used for the determination of TGF β s and their receptors.

MATERIALS AND METHODS

Tissue samples

As one problem associated with studies in keloid patients is a proper definition of keloid and hypertrophic or normal scarring, the selection of patients was performed by experienced dermatologists (O.B. and U.M.). Ethical approval was obtained before the start of the study. Patients gave informed consent prior to their participation; 5-mm punch biopsies were taken. Age-, gender- and site-matched skin from healthy people, hypertrophic scar patients and keloid patients were obtained in the same way.

Primary skin fibroblast cultures

Primary human fibroblast cultures were prepared from fresh tissue. 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 fetal calf serum (FCS; PAA Laboratories, Linz, Austria) and 25 mMol/l N-2-hydroxyethylpiperazine-N'-2-ethanesulphonic acid (HEPES) (Cell concepts). Cells were incubated at 37°C in a 5% (v/v) CO₂ humidified atmosphere. Cells from passages two to four were used for the experiments described. For experiments cells were trypsinized and transferred to phosphate-buffered saline (PBS).

Stimulation of cultured skin fibroblasts with TGF β 1

After washing twice with PBS, subconfluent fibroblast cultures were incubated with various concentrations of TGF β 1 (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 control.

Primer sequences

The primer sequences used for real-time PCR were as follows: for TGF β 1 forward primer: 5'-TGG CGA TAC CTC AGC AAC C-3' (nucleotide position 1379–1398) and reverse primer: 5'-CTC GTG GAT CCA CTT CCA G-3' (nucleotide position 1766–1784) (GenBankTM accession no. X02812, size 405 bp), for TGF β 2 forward primer: 5'-ATC CCG CCC ACT TTC TAC AGA C-3' (nucleotide position 902–924) and reverse primer: 5'-CAT CCA AAG CAC GCT TCT TCC G-3' (nucleotide position 1446–1467) (accession no. M19154, size 565 bp), for TGF β 3 forward primer: 5'-TAC TAT GCC AAC TTC TGC TCA G-3' (nucleotide position 1268–1290) and reverse primer: 5'-AAC TTA CCA TCC CTT TCC TC-3' (nucleotide position 1770–1790) (accession no. X14149, size 522 bp). TGF β RI forward primer: 5'-ACG GCG TTA CAG TGT TCT G-3' (nucleotide position 169–188) and reverse primer: 5'-GGT GTG GCA GAT ATA GAC C-3' (nucleotide position 508–527) (accession no. NM004612, size 358 bp) and for TGF β RII forward primer: 5'-AGC AAC TGC AGC ATC ACC TC-3' (nucleotide position 540–559) and reverse primer: 5'-TGA TGT CTG AGA AGA TGT CC-3' (nucleotide position 1208–1228) (accession no. M85079, size 688 bp).

Real-time PCR

Real-time RT-PCR analyses were performed in a fluorescence temperature cycler (LightCycler, Roche Molecular Biochemicals) according to the manufacturer's instructions. Briefly, total RNA from cultured fibroblasts was isolated using Trizol reagent (Invitrogen), and 2 μ g of total RNA were reverse-transcribed using standard reagents (Invitrogen). The cDNA corresponding to 20 ng of RNA served as a template in a 20- μ l reaction containing 4 mM MgCl₂, 0.5 μ M each primer, and 1 $\times$ LightCycler-FastStart DNA Master SYBR Green I mixture (Roche Molecular Biochemicals). Samples were incubated for an initial denaturing at 95°C for 10 min, followed by 45 cycles, each cycle consisting of 95°C for 15 s, 60°C (touchdown of $-1^\circ\text{C}/\text{cycle}$ from 66–60°C) for 5 s and 72°C for 10 s.

Cycle-to-cycle fluorescence emission readings were monitored at 72°C at the end of each cycle and analysed 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 TGF β and TGF β receptor transcript levels were corrected by normalization based on GAPDH levels.

Melting curves were generated after each run to confirm amplification of specific transcripts. All quantifications were normalized to the housekeeping gene GAPDH that was amplified by intron-spanning primers: GA1, 5'-CCAGCCGAGCCACATCGCTC-3'; and GA2, 5'-ATGAGCCCCAGCCTTCTCCAT-3'. Standard curves were obtained for each primer set with serial dilutions of cDNA.

Immunohistochemistry

Punch biopsies (5 mm) were taken from corresponding sites of the active margin of keloids, hypertrophic scars and normal skin and immediately stored in 0.9% NaCl and snap-frozen in liquid nitrogen. Each biopsy was cut into 5- μ m thin slices, fixed on glass slides and dried at room temperature overnight. The tissue was then fixed for 10 min using acetone. After fixation slides were incubated for 1 h with the primary antibody. Specific antibodies against TGF β RI and II (200 μ g/ml) (polyclonal IgG, rabbit, Santa Cruz) were used for staining at a dilution of 1:200. Following incubation each slide was thoroughly rinsed three times and incubated for 1 h with the secondary antibody (polyclonal anti-goat and anti-rabbit IgG, Santa Cruz). The immunohistochemical procedures were performed according to the labelled streptavidin-biotin method (LSAB, Dako, Carpinteria, CA, USA).

Statistical analysis

Statistical significance between groups was analysed by Student's unpaired *t*-test. All values are expressed as mean $\pm$ SD. Statistical significance was defined as $p \leq 0.05$.

RESULTS

Expression of TGF β mRNA in cultured fibroblasts

Expression of TGF β 1, 2 and 3 mRNA was investigated in cultured fibroblasts from keloids, HS and NS by real-time PCR. TGF β 1 was found to be significantly more highly expressed in keloids than in HS and NS (data not

shown), whereas expression of TGF β 2 was significantly lower in HS ($p < 0.05$) than in keloids and NS. Conversely, the expression of TGF β 3 mRNA in keloid fibroblasts was found to be significantly lower than in HS-derived fibroblasts ($p < 0.01$) (Fig. 1).

Expression of TGF β receptor mRNA in cultured fibroblasts

TGF β R1 and TGF β R2 mRNA expression was investigated in cultured fibroblasts from keloids, NS and HS using real-time PCR (Fig. 2). The expression of TGF β R1 was significantly reduced in HS fibroblasts compared with fibroblasts of NS and keloids. In contrast, TGF β R2 revealed a significantly reduced expression in keloid fibroblasts compared with HS ($p < 0.01$). As a consequence, the ratio of TGF β R1 and TGF β R2 was significantly higher in keloid fibroblasts compared with HS and NS fibroblasts (Fig. 3).

Effect of TGF β 1 stimulation on TGF β R1 and II expression in cultured fibroblasts

The level of TGF β R1 and TGF β R2 mRNA expression in cultured fibroblasts from keloids, HS and NS was determined after stimulation with TGF β 1 (20 ng/ml) for 6 h.

TGF β 1 stimulation showed a tendency to up-regulation of TGF β R1 mRNA expression in HS fibroblasts. In keloid fibroblasts and in NS-derived fibroblasts TGF β R1 mRNA expression showed a tendency to down-regulation (data not shown). TGF β R2 mRNA was in all cases down-regulated by TGF β 1 in keloids and also tended to be down-regulated in NS fibroblasts. In HS TGF β R2 mRNA showed a trend towards up-regulation.

Immunohistochemistry of tissue biopsies of keloids, hypertrophic scars and normal skin

Staining of keloid, NS and HS biopsies with TGF β R1 and II antibodies did not reveal a different expression

pattern (data not shown). No specific staining in dermal cells or fibroblasts was found.

DISCUSSION

TGF β 1 mRNA expression was found to be higher in keloids than in HS and NS fibroblasts. These results are consistent with findings from Lee et al. (7). Our data further showed that TGF β 2 mRNA expression was significantly increased in cultured fibroblasts from keloids as compared with HS (Fig. 1). In contrast TGF β 3 mRNA expression was significantly lower in keloids. Ogawa et al. (16) found that TGF β 3 reduces connective tissue deposition. Therefore our results suggest a possible role of reduced TGF β 3 expression in the formation of keloids. The high expression of TGF β 3 in HS might reflect well-controlled fibrosis. This might be a reason why HS do not have a tendency to grow invasively. This is consistent with the finding that TGF β 3 mRNA is strongly induced at later stages of the wound repair process (17). In addition TGF β 3 is undergoing clinical trials for the prevention of scarring (18), underlining the role of TGF β 3 as a fibrosis inhibitor that is decreased in keloids.

In contrast to TGF β 3, TGF β 2 expression is increased in keloids compared with HS (Fig. 1). These findings are consistent with those of Wang et al. (19), suggesting that TGF β 2 might act as a fibrosis promoter. However, Lu et al. (8) showed an increased expression of TGF β 1 and 2 and a decreased expression of TGF β 3 in hypertrophic scars using in situ hybridization. The different results might be attributed to the different methods used or to differences in age and biopsy site of skin specimens. This underlines the importance of standardized conditions (skin biopsies, cell culture) when investigating TGF β . Interestingly, TGF β 2 mRNA is expressed at the same level in keloids as in NS (Fig. 1). According to the literature (7) a lower level of TGF β 2 mRNA expression is expected in NS compared with keloids. Our findings

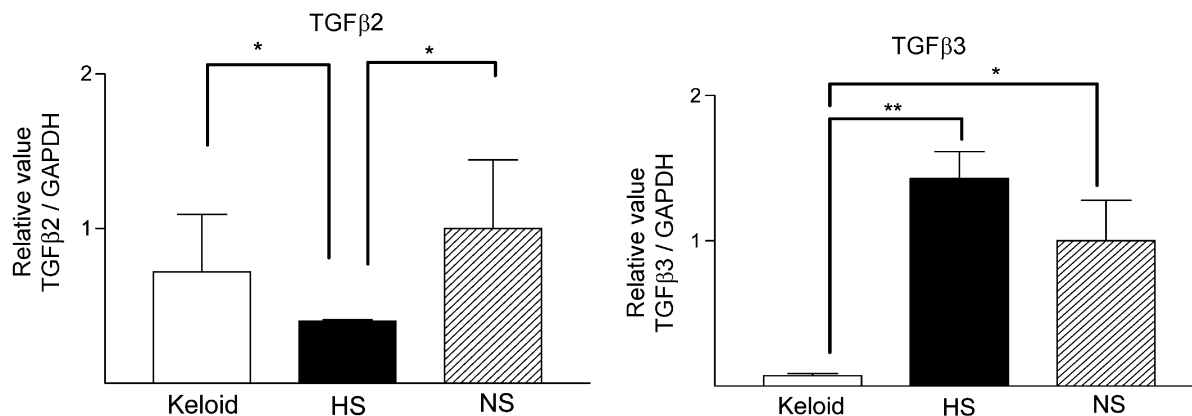


Fig. 1. Quantitative TGF β 2 and TGF β 3 mRNA expression in subconfluent cultured fibroblasts from keloids, hypertrophic scars (HS) and normal skin (NS) analysed by real-time PCR using the LightCycler technique ($n=5$). The data represent mean $\pm$ SD. * $p < 0.05$ and ** $p < 0.01$.

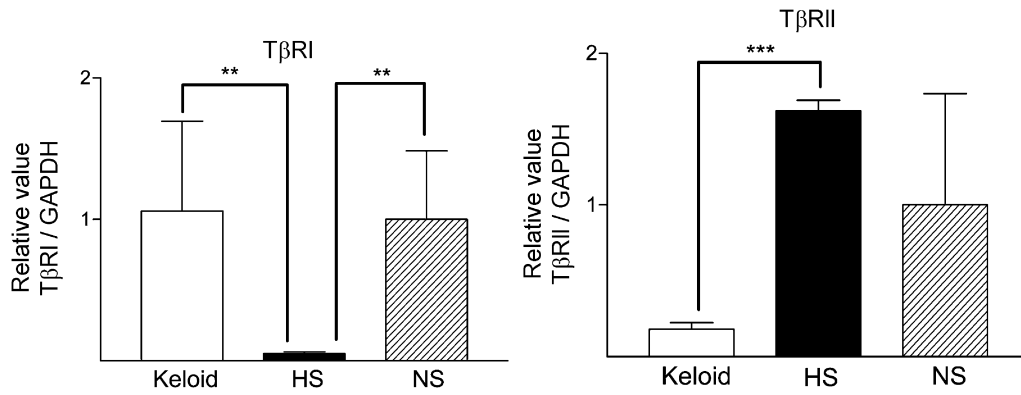


Fig. 2. TGF β RI (T β RI) and TGF β RII (T β RII) mRNA expression in subconfluent cultured fibroblasts from keloids, hypertrophic scars (HS) and normal skin (NS) analysed by quantitative real-time PCR using the LightCycler technique ($n=5$). The data represent mean $\pm$ SD. ** $p < 0.01$ and *** $p < 0.001$.

might lead to the presumption that TGF β 2 alone does not induce fibrosis. It is more likely that a certain combination of TGFs and their receptors may lead to increased extracellular matrix synthesis.

TGF β exerts biological effects by interacting with two types of transmembrane receptors (types I and II) possessing protein serine/threonine kinase activity (20). The type II receptor is involved in initial ligand binding. Once the ligand is bound, the type II receptor binds to a type I receptor forming a complex; type I receptor is then phosphorylated by the kinase domain of type II receptor, resulting in a transduction of down-stream signals. The expression of two types of TGF β receptors was shown at high levels in normal and wounded skin (17).

We found (Fig. 2) that the mRNA expression of TGF β RI was significantly decreased in HS compared with keloids and NS, whereas TGF β RII mRNA expression was significantly decreased in keloid

fibroblasts as compared with HS but not compared to NS. Goldberg et al. (21) showed that over-expression of TGF β RII leads to inhibition of fibroblast proliferation. The results imply that reduced TGF β RII expression leads to keloid formation via increased fibrosis. Chin et al. (22) found an increased expression of TGF β RI and II in keloids. At later stages of keloid development an increased expression of TGF β RII may lead to decreased keloid activity and growth. This is consistent with the clinical finding that older keloids often are less itchy and less painful. Varying ages and clinical stages of keloids from which biopsies were taken might be the cause of different results in TGF β RII expression. Furthermore, the correct clinical definition of keloids compared to HS is important.

Recent studies suggest that the ratio of type I to type II receptor may determine the biological effect of TGF β on proliferation and target gene expression (23). For example, an increased type I/type II ratio leads to enhanced collagen synthesis by TGF β . In support of a role of TGF β receptors in keloid pathogenesis, we found that the ratio of TGF β RI/TGF β RII mRNA expression was significantly increased in keloids as compared with HS and NS (Fig. 3). A combination of increased TGF β 1 and 2, decreased TGF β 3, and increased TGF β RI/TGF β RII ratio might lead to unlimited collagen over-expression. However, it could also be that over-expression of collagen and other extracellular matrix proteins leads to an increased TGF β RI/TGF β RII ratio.

The TGF β RI/TGF β RII ratio is also increased in NS compared with HS. Speculatively, the increased TGF β 3 expression and decreased TGF β 1 in NS compared with keloids prevent the over-synthesis of extracellular matrix. HS is also characterized by collagen over-expression. Our results showed a low TGF β RI/TGF β RII ratio in HS. As mentioned above, this should lead to inhibition of matrix synthesis. This conflicts with the clinical finding of increased collagen seen in HS. In contrast to keloids HS, do not grow invasively into the

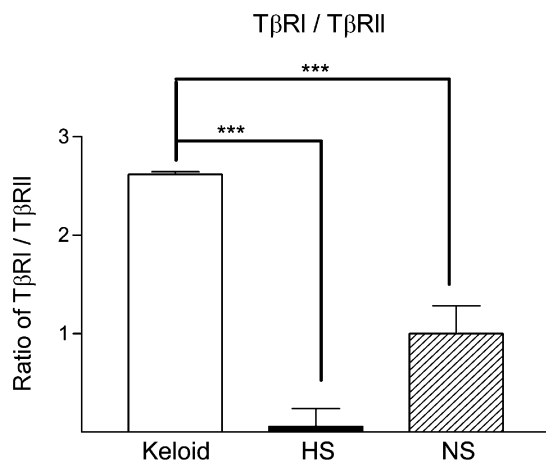


Fig. 3. Ratio of TGF β RI (T β RI) and TGF β RII (T β RII) mRNA expression in subconfluent cultured fibroblasts from keloids, hypertrophic scars (HS) and normal skin (NS) analysed by quantitative real-time PCR using the LightCycler technique ($n=5$). The data represent mean $\pm$ SD. *** $p < 0.001$.

surrounding healthy skin, but stop growing after a while. The decreased TGF β RI/TGF β RII ratio in HS might reflect the inhibition of extracellular matrix production in HS. In order to clarify this point one would need to investigate the TGF β RI/TGF β RII ratio in newly developing HS.

We also investigated the response of cultured fibroblasts to TGF β 1 stimulation to determine whether there is an altered response of TGF β RI and TGF β RII expression in keloids and HS. TGF β 1-stimulation showed a trend to down-regulation of TGF β RI and TGF β RII mRNA expression in keloids and in NS (data not shown). In contrast, TGF β 1 stimulation induced up-regulation of TGF β RI and TGF β RII mRNA expression in HS fibroblasts. Even though these findings were not of statistical significance they did show a trend in regulation. The TGF β RI/TGF β RII ratio was not changed in keloid fibroblasts after TGF β 1 stimulation but was found to increase in HS fibroblasts. This might indicate that HS fibroblasts are able to promote fibrosis after stimulation, whereas keloid fibroblasts do not change their phenotype after stimulation and persist at a high level of activation.

Immunohistochemistry staining showed no difference in expression of TGF β RI in keloids, HS and NS. The staining with antibodies against TGF β RII showed a stronger staining in the basal epidermal layer in keloids and HS compared with NS (data not shown). No staining of the basal cell layer in NS was found. Interestingly, no fibroblasts in keloids, HS or NS showed a specific staining pattern. We could only show staining in the epidermal cell layer. Further experiments are needed to more closely determine the role of TGF β RII expression in epidermal cells in keloid scarring.

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