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 (TGFβ) is involved in keloid formation. Therefore we investigated the expression of TGFβ1, 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 TGFβ1, 2 and 3 and of TGFβ receptors I and II (TGFβRI and II) was analysed by real-time PCR using the Lightcycler technique. Our data demonstrate significantly lower TGFβ2 mRNA expression in hypertrophic scar fibroblasts as compared with fibroblasts derived from keloids and normal skin (p < 0.05). In contrast, TGFβ3 mRNA expression was significantly lower in keloid fibroblasts in comparison with fibroblasts derived from hypertrophic scar and normal skin (p < 0.01). TGFβRI mRNA expression was significantly decreased in hypertrophic scar fibroblasts (p < 0.01) and TGFβRII mRNA expression was decreased in keloids compared with hypertrophic scar fibroblasts (p < 0.001). The ratio of TGFβRI/TGFβRII expression was increased in keloids compared with hypertrophic scar and normal skin fibroblasts. As recently supposed, an increased TGFβRI/TGFβRII ratio could promote fibrosis. Therefore our data support a possible role of TGFβRI and TGFβRII in combination with a certain TGFβ expression pattern as fibrosis-inducing factors in keloids. Key words: wound healing; fibroblasts; TGFβ receptors.

(Accepted September 20, 2004.)

Acta Derm Venereol 2005; 85: 216–220.

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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 TGFβ isoforms (TGFβ1, TGFβ2 and TGFβ3) with similar but not identical bioactivities have been described. TGFβ1 and TGFβ2 proteins were recently found to be more highly expressed in keloid fibroblasts than in normal human dermal fibroblast cultures (7). Furthermore, TGFβ1, 2 and TGFβRI (TGFβ receptor I) were found to be increased in HS compared with normal skin (NS). In contrast, TGFβ3 and TGFβRII expression were decreased in HS compared with normal skin (8).

Keloid fibroblasts show a unique sensitivity to TGFβ. Treatment with TGFβ1 results in stimulation of total protein synthesis in normal dermal fibroblasts but not in keloid fibroblasts. TGFβ1-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 TGFβ1 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 TGFβ expression in keloid patients have not demonstrated TGFβ gene polymorphisms (14, 15).

The purpose of the present study was to investigate whether there was an aberrant expression of TGFβ1, 2 and 3 or of the two surface receptors for TGFβ (TGFβRI and II). Fibroblast cultures were established.
from freshly taken skin biopsies and further used for the determination of TGFβs 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 human 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 TGFβ1
After washing twice with PBS, subconfluent fibroblast cultures 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 control.

Primer sequences
The primer sequences used for real-time PCR were as follows:

- For TGFβ1 forward primer: 5’-TGG CGA CAT TCT AGC AAC ACG 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 TGFβ2 forward primer: 5’-ATC CCG CCC ACT TAC AGA C-3’ (nucleotide position 902–924) and reverse primer: 5’-CAT CAA AAG CAC GCT TCT TCC G-3’ (nucleotide position 1446–1467) (accession no. M19154, size 358 bp), for TGFβ3 forward primer: 5’-TAC TAT TAT GCC AAC TTC TGC TCA G-3’ (nucleotide position 1268–1290) and reverse primer: 5’-AAC TTA TCA TCC CTT TCC TC-3’ (nucleotide position 1770–1790) (accession no. X14149, size 322 bp), TGFβRI forward primer: 5’-ACG GCC TTA CAG TGT TCT TCT-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 TGFβRII forward primer: 5’-AAC TAC 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 μg 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-μl reaction containing 4 mM MgCl2, 0.5 μM each primer, and 1 × 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°C/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 TGFβ1 and TGFβ 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’-CAAGCCGAGCCACATCGCTC-3’; and GA2, 5’-ATGAGCCCCAGGCTTCTCCAT-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-μm 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 TGFβRI and II (200 μg/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 ± SD. Statistical significance was defined as p ≤ 0.05.

RESULTS
Expression of TGFβ mRNA in cultured fibroblasts
Expression of TGFβ1, 2 and 3 mRNA was investigated in cultured fibroblasts from keloids, HS and NS by real-time PCR. TGFβ1 was found to be significantly more highly expressed in keloids than in HS and NS (data not
shown), whereas expression of TGFβ2 was significantly lower in HS (p < 0.05) than in keloids and NS. Conversely, the expression of TGFβ3 mRNA in keloid fibroblasts was found to be significantly lower than in HS-derived fibroblasts (p < 0.01) (Fig. 1).

Expression of TGFβ receptor mRNA in cultured fibroblasts

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

Effect of TGFβ1 stimulation on TGFβRI and II expression in cultured fibroblasts

The level of TGFβRI and TGFβRII mRNA expression in cultured fibroblasts from keloids, HS and NS was determined after stimulation with TGFβ1 (20 ng/ml) for 6 h. TGFβ1 stimulation showed a tendency to up-regulation of TGFβRI mRNA expression in HS fibroblasts. In keloid fibroblasts and in NS-derived fibroblasts TGFβRI mRNA expression showed a tendency to down-regulation (data not shown). TGFβRII mRNA was in all cases down-regulated by TGFβ1 in keloids and also tended to be down-regulated in NS fibroblasts. In HS TGFβRII 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 TGFβRI and II antibodies did not reveal a different expression pattern (data not shown). No specific staining in dermal cells or fibroblasts was found.

DISCUSSION

TGFβ1 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 TGFβ2 mRNA expression was significantly increased in cultured fibroblasts from keloids as compared with HS (Fig. 1). In contrast TGFβ3 mRNA expression was significantly lower in keloids. Ogawa et al. (16) found that TGFβ3 reduces connective tissue deposition. Therefore our results suggest a possible role of reduced TGFβ3 expression in the formation of keloids. The high expression of TGFβ3 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 TGFβ3 mRNA is strongly induced at later stages of the wound repair process (17). In addition TGFβ3 is undergoing clinical trials for the prevention of scarring (18), underlining the role of TGFβ3 as a fibrosis inhibitor that is decreased in keloids.

In contrast to TGFβ3, TGFβ2 expression is increased in keloids compared with HS (Fig. 1). These findings are consistent with those of Wang et al. (19), suggesting that TGFβ2 might act as a fibrosis promoter. However, Lu et al. (8) showed an increased expression of TGFβ1 and 2 and a decreased expression of TGFβ3 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 TGFβ. Interestingly, TGFβ2 mRNA is expressed at the same level in keloids as in NS (Fig. 1). According to the literature (7) a lower level of TGFβ2 mRNA expression is expected in NS compared with keloids. Our findings
might lead to the presumption that TGFβ2 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.

TGFβ 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 TGFβ receptors was shown at high levels in normal and wounded skin (17).

We found (Fig. 2) that the mRNA expression of TGFβRI was significantly decreased in HS compared with keloids and NS, whereas TGFβRII 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 TGFβRII leads to inhibition of fibroblast proliferation. The results imply that reduced TGFβRII expression leads to keloid formation via increased fibrosis. Chin et al. (22) found an increased expression of TGFβRI and II in keloids. At later stages of keloid development an increased expression of TGFβRII 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 TGFβRII 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 TGFβ on proliferation and target gene expression (23). For example, an increased type I/type II ratio leads to enhanced collagen synthesis by TGFβ. In support of a role of TGFβ receptors in keloid pathogenesis, we found that the ratio of TGFβRI/TGFβRII mRNA expression was significantly increased in keloids as compared with HS and NS (Fig. 3). A combination of increased TGFβ1 and 2, decreased TGFβ3, and increased TGFβRI/TGFβRII 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 TGFβRI/TGFβRII ratio.

The TGFβRI/TGFβRII ratio is also increased in NS compared with HS. Speculatively, the increased TGFβ3 expression and decreased TGFβ1 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 TGFβRI/TGFβRII 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
surrounding healthy skin, but stop growing after a while. The decreased TGFβRI/TGFβRII 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 TGFβRI/TGFβRII ratio in newly developing HS.

We also investigated the response of cultured fibroblasts to TGFβ1 stimulation to determine whether there is an altered response of TGFβRI and TGFβRII expression in keloids and HS. TGFβ1-stimulation showed a trend to down-regulation of TGFβRI and TGFβRII mRNA expression in keloids and in NS (data not shown). In contrast, TGFβ1 stimulation induced up-regulation of TGFβRI and TGFβRII mRNA expression in HS fibroblasts. Even though these findings were not of statistical significance they did show a trend in regulation. The TGFβRI/TGFβRII ratio was not changed in keloid fibroblasts after TGFβ1 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 TGFβRI in keloids, HS and NS. The staining with antibodies against TGFβRII 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 TGFβRII expression in epidermal cells in keloid scarring.

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