in vivo *19*: 959-964 (2005)

Comparison of Transforming Growth Factor-β/Smad Signaling between Normal Dermal Fibroblasts and Fibroblasts derived from Central and Peripheral Areas of Keloid Lesions

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Abstract. *Background: Keloids are benign proliferations of fibroblasts, but their exact etiology and molecular pathogenesis are unknown. Materials and Methods: Fibroblasts were isolated from the central and peripheral regions of keloids, and the growth behavior and molecular characteristics of the keloid fibroblasts were compared with those of age-adjusted normal dermal fibroblasts. Results: Central (but not peripheral) keloid fibroblasts exhibited significantly increased growth and high levels of expression of transforming growth factor-beta (TGF-β) receptor 1 and Smad 2/3. Conclusion: Proliferation of central keloid fibroblasts, which results in keloid formation, appears to mainly involve the TGF-β/Smad pathway.*

Keloid is a disorder that occurs in wounds (which may be clinically inapparent), and has only been found in humans. Keloids are characterized by abnormal fibroblast proliferation and overproduction of extracellular matrix (which represent pathological wound healing) that grows beyond the wounded area (1). Keloids do not regress spontaneously, are resistant to treatment and tend to recur after excision. Several growth factors have been implicated in the pathogenesis of keloids, including transforming growth factor-beta (TGF-β), epidermal growth factor, fibroblast growth factors and platelet-derived growth factor (2). TGF-β and TGF-β signaling appear to play key roles in keloid formation (1-5). However, the molecular mechanisms underlying keloid formation are unclear. Recent reports about keloid fibroblasts have focused on the TGF-β/Smad signaling pathway (4, 6). However, there have been no precise studies of differences in behavior of keloid fibroblasts between different locations in keloid lesions. In the present study, fibroblasts were isolated from the center and periphery of 3 keloids in 3 patients, and their proliferative activity and TGF-β/Smad signaling compared with those of normal dermal fibroblasts from 3 age-matched individuals.

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Key Words: Keloid, fibroblasts, TGF-β, Smad.

Materials and Methods

Cell culture. The study protocol was approved by the ethics committee of our institutions (Kansai Medical University and Kishiwada Municipal Hospital, Japan), and informed consent was obtained from all subjects. Clinically typical, untreated keloid tissue samples were obtained from 3 patients using standard surgical procedures, and normal age-adjusted skin samples were obtained from 3 individuals without keloids. All subjects were Japanese, and ranged in age from 17 to 54 years (mean±SE: 35±18 years for keloid patients, and 34±18 years for normal individuals; Table I). Primary fibroblast cultures were made using the explant method. Briefly, explant tissue samples were cut into small pieces that were washed twice in PBS(–), placed in culture dishes (Nunc, Naperville, CT, USA) and cultured in Dulbecco's modified Eagle's minimum essential medium (DMEM 0/5921, Sigma, St. Louis, MO, USA), supplemented with 10% fetal bovine serum (FBS, Gibco BRL, Grand Island, NY, USA) and 1% stabilized antibiotic-antimycotic solution (Sigma), in a humidified incubator (37˚C and 5% CO_2). The early passages (third to fifth) of cells were used for the following experiments.

Viable cell assay. Viable central keloid fibroblasts (c-KF), peripheral keloid fibroblasts (p-KF) and normal fibroblasts (NF) were counted using a colorimetric 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazoleum bromide (MTT) assay. Initially, $1x10^4$ cells were seeded in 24-well culture plates and cultured in DMEM with 10% FBS. At fixed intervals (2, 6, 8 and 10 days), 100 μl of MTT solution (Sigma) was added into each well and the plates were then incubated for 3 h. Next, the medium was replaced with 1000 μl of dimethylsulfoxide and absorbance at 540 nm was read using a plate reader.

Table I. *Data for keloid and normal samples.*

Sample	Age (years)	Sex	Site
KF1	53	F	Abdomen
KF2	36	F	Upper arm
KF3	17	M	Chest
NF1	54	F	Face
NF2	33	F	Back
NF3	17	F	Forearm

KF; keloid fibroblast, NF; normal fibroblast.

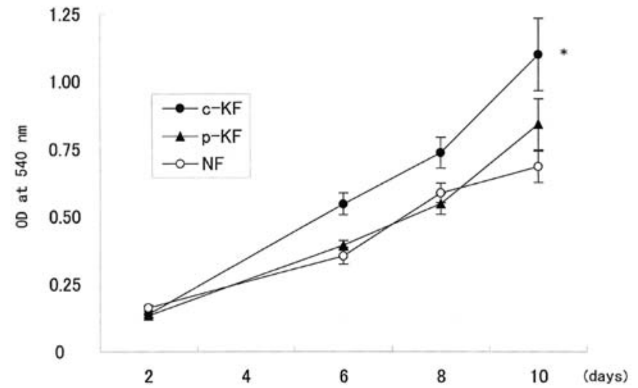

Figure 1. *Proliferation of central keloid fibroblasts (c-KF) and peripheral keloid fibroblasts (p-KF), compared to normal dermal fibroblasts (NF). The data represents means of 3 samples. * p<0.05, compared with NF at the same time-point.*

Western blotting. To examine growth signaling, fibroblast expression of the proteins TGF-β receptor 1, TGF-β receptor 2, Smad 2/3 and Smad 4 was examined by Western blotting. Cells were cultured in DMEM with 10% FBS. When the cells became semi-confluent, the culture medium was changed to DMEM without FBS, followed by incubation for another 24 h. Then, the cells were trypsinized with 0.25% trypsin-EDTA solution, washed in PBS(–), and lysed with lysis buffer containing SDS. Immunoblotting was performed as previously described (7). Briefly, cell lysates were prepared, and aliquots of each lysate (100 μg of protein) were electrophoresed on SDS-polyacrylamide gels and blotted onto nitrocellulose membrane (Hybond-P, Amersham Pharmacia Biotech, Buckinghamshiere, UK). The membranes were probed with anti-TGF-β receptor 1 antibody (clone V-22, Santa Cruz Biotechnology, Santa Cruz, CA, USA), anti-TGF-β receptor 2 antibody (clone C-16, Santa Cruz), anti-Smad 2/3 antibody (clone-18, BD Biosciences, San Jose, CA, USA), anti-Smad 4 antibody (clone 49, BD Biosciences) or anti-actin antibody (clone C-11, Santa Cruz). Next, the membrane was treated with peroxidase-conjugated goat anti-mouse or anti-rabbit IgG antiserum (Envision + system HRP, DakoCytomation A/S, Glostrup, Denmark). The detection of immobilized antigen was performed using ECL Plus Western blotting detection reagents and hyperfilm (Amersham Pharmacia Biotech), according to the manufacturer's instructions. The intensity of the bands was quantified, and the relative expression (compared with actin expression) was calculated.

Data analysis. All results were expressed as the mean±SE, and statistical significance was assessed using Fisher's protected least significant difference test, after a non-repeated measure ANOVA parametric test. Differences with a p-value of <0.05 were considered significant.

Results

Proliferation of fibroblasts from keloids and normal skin. During 10 days of culture, the proliferation of peripheral keloid fibroblasts (mean of p-KF1, 2 and 3) was similar to that of age-adjusted normal fibroblasts (mean of NF1, 2 and 3). In contrast, the proliferation of central keloid fibroblasts (means of c-KF1, 2 and 3) was much faster than that of normal fibroblasts, and the cell number of central keloid fibroblasts was significantly greater than that of normal fibroblasts at 10 days ($p<0.05$; Figure 1).

TGF-β receptor 1 and receptor 2 protein expression in fibroblasts from keloids and normal skin. The representative TGF-β receptor 1 expression of NF3 and KF3 and quantifications of 3 individual experiments are shown in Figure 2a and b, respectively. The expression of TGF-β receptor 1 protein was low in NF3 and p-KF3, whereas its expression was high in c-KF3, as shown by the corresponding bands with an approximate molecular weight of 53 kDa ($p<0.05$, respectively). In contrast, NF3, c-KF3 and p-KF3 all had similar levels of expression of TGF-β receptor 2 protein (approximate molecular weight, 70 kDa) (Figure 2b). A similar tendency was exhibited by the other 2 NFs, c-KFs and p-KFs (KF1 and 2 *vs*. NF1 and 2) (data not shown).

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Figure 2. *TGF-β receptor 1 and receptor 2 expression in normal fibroblasts (NF3) and keloid fibroblasts (KF3) from the center and periphery of the lesion. NF, normal fibroblasts; c-KF, central keloid fibroblasts; p-KF, peripheral keloid fibroblasts. a. Representative data for TGF-β receptor 1 expression. b. Levels of TGF-β receptor 1 and receptor 2 protein, relative to actin staining, after 3 different experiments (means±SE). Mean NF value was set to 1.0. * p<0.05.*

Figure 3. *Smad 2/3 and 4 expression in normal fibroblasts (NF3) and keloid fibroblasts (KF3) from the center and periphery of the lesion. a. Representative data for Smad 2/3 expression. b. Levels of Smad 2/3 and 4 protein, relative to actin staining, after 3 different experiments (mean±SE). Mean NF value was set to 1.0. *p<0.05.*

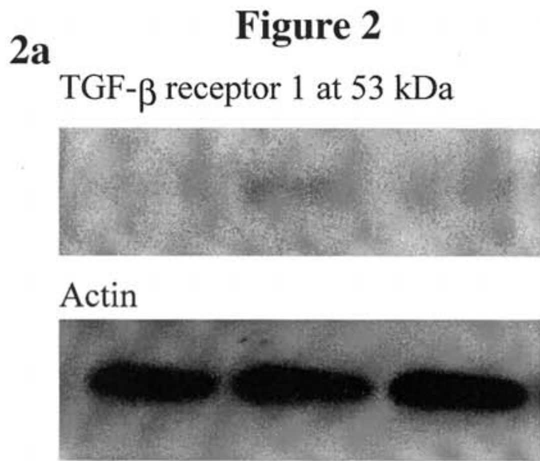


Figure 2
2a
TGF-β receptor 1 at 53 kDa
Actin

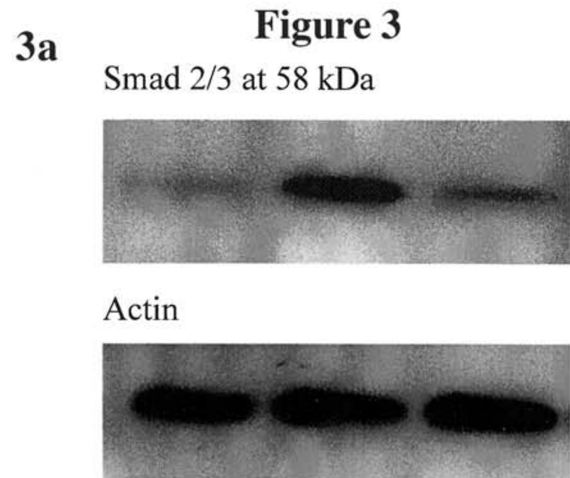
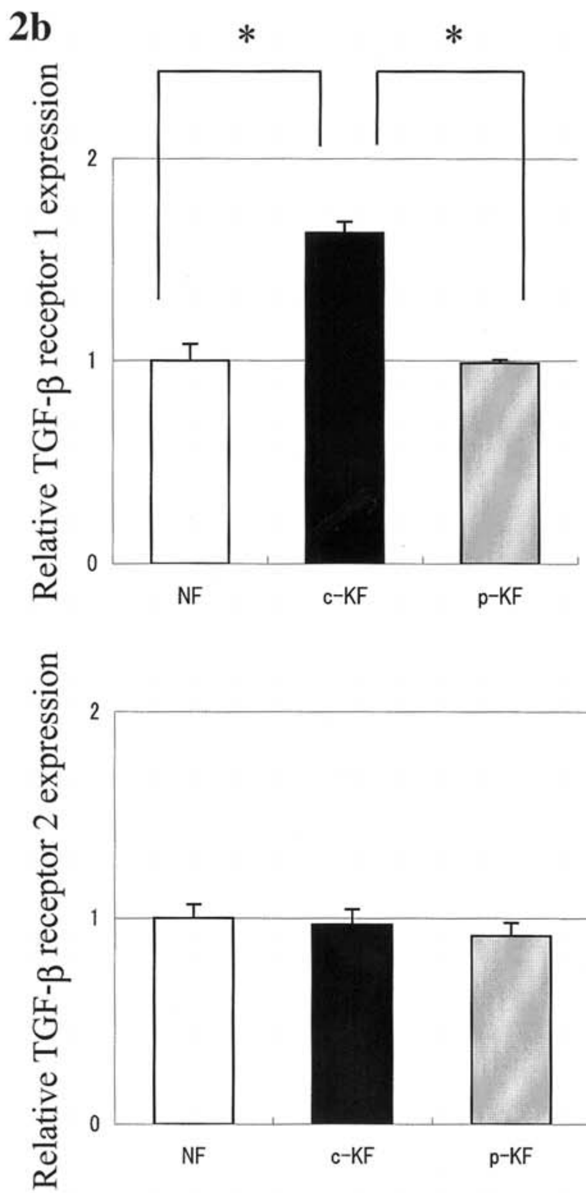
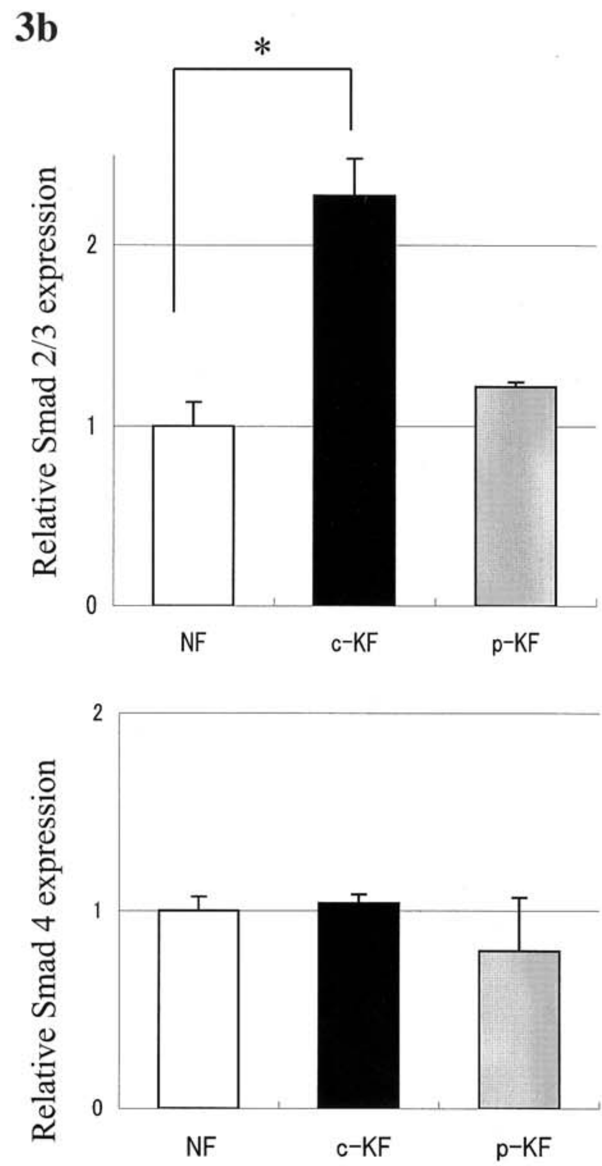


Figure 3
3a
Smad 2/3 at 58 kDa
Actin


2b
*
*
Relative TGF-β receptor 1 expression
2
1
0
NF
c-KF
p-KF
Relative TGF-β receptor 2 expression
2
1
0
NF
c-KF
p-KF


3b
*
Relative Smad 2/3 expression
2
1
0
NF
c-KF
p-KF
Relative Smad 4 expression
2
1
0
NF
c-KF
p-KF

Smad 2/3 and Smad 4 protein expression in fibroblasts from keloids and normal skin. Smad 2/3 expression was significantly higher in c-KF3 than in NF3 ($p<0.05$) and p-KF (Figure 3a and b). In contrast, NF3, c-KF3 and p-KF3 all had similar levels of expression of Smad 4 (Figure 3b). A similar tendency was exhibited by the other 2 NFs, c-KFs and p-KFs (data not shown).

Discussion

Keloid is a unique proliferative disorder of fibroblasts that occurs in wounds. Keloids grow beyond the original wound and do not regress spontaneously (1). They exhibit an abnormal balance between cell proliferation and cell death (8). Immunohistochemical staining of proliferative cell nuclear antigen (PCNA) has revealed that the fibroblast density and proliferative activity are higher in keloids than in normal dermis *in vivo* (9). Several types of fibroblasts are present in keloids; fibroblasts derived from the central region of keloid lesions proliferate faster than normal fibroblasts *in vitro*, whereas fibroblasts derived from other regions of keloids proliferate at the same rate as normal fibroblasts (8, 10). These findings are consistent with the present observation that fibroblasts from the central (but not peripheral) part of keloids grew faster than normal fibroblasts *in vitro*; central keloid fibroblasts appear to play a key role in keloid proliferation.

Keloid-derived fibroblasts have a greater mitogenic response to TGF-β than normal cells (3). TGF-β-related signaling is thought to be a cause of keloid formation (4, 11, 12). Keloid-derived fibroblasts exhibit unique sensitivity to TGF-β (3). There are 3 isoforms of TGF-β (β1, β2 and β3). The TGF-β family members exert a wide range of biological effects on a large variety of cells. Those effects include regulation of cell growth, differentiation, matrix production and apoptosis. TGF-β1 and -β2 are believed to promote fibrosis and scar formation. TGF-β1 and -β2 ligands are expressed at greater levels in keloid-derived fibroblasts than in normal human dermal fibroblasts (4). Also, keloid-derived fibroblasts exhibit increased production of TGF-β1 and -β2, which is consistent with the hypothesis that TGF-β1 and -β2 play roles in the pathogenesis of keloid formation (13). Moreover, neutralizing antibody against pan-TGF-β or TGF-β1 (but not -β2) can suppress the proliferation of keloid-derived fibroblasts (12, 14). The cellular activity of TGF-βs is initiated when they bind to their receptors; type 1 and type 2 receptors are involved in TGF-β signaling, and type 3 receptor presents ligands to type 1 and 2 receptors (15, 16). Then, the downstream effector molecules, including members of the Smad family, are activated (15, 16). Smads are intracellular TGF-β signaling molecules; their name is derived from *Sma* and *Mad*. In a previous study, keloid-derived fibroblasts exhibited increased expression of TGF-β receptors 1 and 2, compared with normal fibroblasts (4). However, in another study, the expression of the TGF-β receptor 1 was elevated, but expression of the TGF-β receptor 2 was not (12). Activated TGF-β type 1 receptor phosphorylates Smad 2 and 3, which then bind to Smad 4. The resulting Smad complex then moves to the nucleus (17), where it activates target gene transcription in association with DNA-binding partners (16). There have been reports of up-regulation of Smad 2 and/or 3 in keloid-derived fibroblasts (4, 12). The present results showed up-regulation of TGF-β receptor 1 and Smad 2/3 in central keloid fibroblasts (but not peripheral keloid fibroblasts), compared with normal fibroblasts; activation of the TGF-β signaling pathway in central keloid fibroblasts appears to be essential for keloid formation. Recent studies show that blocking of TGF-β/Smad signaling represents a potential drug target for keloid reduction therapy (16, 18).

In conclusion, central keloid fibroblasts (but not peripheral keloid fibroblasts) proliferate more rapidly than normal dermal fibroblasts and express TGF-β receptor 1 and Smad 2/3 proteins at higher levels than normal dermal fibroblasts, indicating that keloid formation is caused by up-regulation of the TGF-β/Smad signaling pathway. Thus, blocking of the TGF-β /Smad pathway may be clinically useful for the treatment of keloids.

Acknowledgements

The authors wish to thank Ms T. Akamatsu for her technical assistance and Ms S. Nakagawa for preparing the manuscript.

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Received May 23, 2005
Accepted August 16, 2005