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INVESTIGATIVE REPORT

Upregulation of the Wnt/β-catenin Pathway Induced by Transforming Growth Factor-β in Hypertrophic Scars and Keloids

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Hypertrophic scars and keloids represent a dysregulated response to cutaneous wounds, which results in an excessive deposition of collagen. Transforming growth factor-β (TGF-β) is the key regulator in the pathogenesis of fibrosis. Accumulating evidence suggests that Wnt signalling and its effector β-catenin also play an important role in wound healing. The role of Wnt/β-catenin signalling in TGF-β induced collagen deposition in hypertrophic scars and keloids was studied. Transcriptional assays and Western blotting was performed using fibroblast cell lines established from normal skin and hypertrophic scar tissue. Immunohistochemical studies were performed using scar tissues. We provide evidence that TGF-β induces activation of β-catenin mediated transcription in human dermal fibroblasts via the Smad3 and p38 MAPK pathways. Immunohistochemical studies demonstrated that β-catenin protein levels are elevated in hypertrophic scar and keloid tissues. This finding may be relevant to the pathogenesis of hypertrophic scars and keloids. ***Key words: Smad3; p38 MAPK; fibrosis; wound healing.***

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Signalling by transforming growth factor-β (TGF-β) and Wnt can regulate cell fate and proliferation during development and tissue maintenance (1, 2). TGF-β is a multifunctional growth factor that plays a critical role as a regulator of immune cell function, epithelial cell growth, and extracellular matrix (ECM) deposition (3). Overproduction of TGF-β is associated with excessive deposition of scar tissue and fibrosis (3). TGF-β signals through a heteromeric receptor complex of type I and II receptor serine/threonine kinases. The signal is propagated downstream through Smads, a family of evolutionarily conserved intracellular mediators that convey information from the cell membrane into the nucleus (2). TGF-β can also activate the extracellular signal regulated kinase (ERK), c-jun N-terminal or stress activated protein kinases (JNK) and p38 mitogen-activated protein kinase (MAPK) (4). In human dermal fibroblasts, TGF-β also transduces signals through the p38 MAPK pathway to stimulate collagen production (5).

β-catenin is a mediator in the canonical Wnt (wingless) signalling pathway, which has a pivotal role in embryonic development and neoplasia (1). Wnt signalling is initiated by secreted Wnt proteins, which bind to the Frizzled family of receptors (1). The activation of the receptor leads to the stabilization of cytosolic β-catenin by inhibiting glycogen-synthase kinase-dependent phosphorylation of β-catenin and results in an increase in β-catenin protein levels. The accumulated β-catenin binds T-cell factor/lymphoid enhancer binding factor (TCF/LEF) transcription factors and activates transcription of target genes. It is already known that β-catenin functions during fibroproliferative processes such as aggressive fibromatosis. Furthermore, Wnt signalling and its effector β-catenin are suggested to be the key regulators in wound healing (6, 7). Moreover, recent studies have shown co-operation between TGF-β and Wnt signalling pathways in controlling certain developmental events in *Xenopus* and *Drosophila* (8, 9).

Hypertrophic scars and keloids represent a dysregulated response to cutaneous wounding, resulting in an excessive deposition of ECM, especially collagen. TGF-β is believed to be responsible for excessive ECM deposition in hypertrophic scars, keloids and other fibrotic conditions (10). Since β-catenin is known to accumulate during fibroproliferation (6, 7), we speculated that it could play a role in the mechanisms that lead to hypertrophic/keloid scarring. We hypothesized that TGF-β may convey signals to induce activation of β-catenin signalling, which might be responsible for hypertrophic/keloid scarring. In this study, a novel role of Wnt/β-catenin signalling in TGF-β induced collagen deposition in hypertrophic scars and keloids was examined.

MATERIALS AND METHODS

Cell cultures

The detailed profiles of cell strains of fibroblasts used in this study are described elsewhere (11). Hypertrophic scar cell lines used were a cell line established from hypertrophic scar tissue obtained from an 11-year-old female (HS1) and a cell line established from hypertrophic scar tissue obtained from a 17-year-old female (HS2). Control fibroblast cell lines were a cell line established from normal tissue obtained from an 18-year-old female (N1) and a cell line established from normal tissue obtained from a 20-year-old female (N2), respectively. Explant tissue was dissociated enzymatically by 0.25% collagenase type I (Sigma, St Louis, MO, USA) and 0.05% DNase (Sigma)

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in DMEM with 20% foetal bovine serum (FBS) (GibcoBRL, Grand Island, NY, USA) (5). Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS. Fibroblasts frozen in early passages were thawed and used for experiments. Hypertrophic scar cell lines were confirmed to have increased type I and III collagen production and decreased collagenase production compared with control fibroblasts (11).

Plasmid constructs, transient transfection and luciferase assay

The plasmid pTOPFLASH, and pFOPFLASH contain three copies of the optimal TCF-binding motif CCTTTGATC upstream of a minimal c-Fos promoter driving luciferase expression and mutated binding motif, respectively (12). The expression vectors for Smad3 and p38α are described elsewhere (5, 13). For transient transfection, 60–80% confluent cells in 6-well plates were transfected using FuGENE 6 transfection reagents (Roche Molecular Biochemicals, Indianapolis, IN, USA) following the manufacturer's recommendations. Twenty-hours post-transfection, cells were stimulated with 5 ng/ml TGF-β1 (R&D Systems, Minneapolis, MN, USA) for an additional 24 h in culture. In some experiments, 10 μmol/L SB203580 (Biomol, Plymouth Meeting, PA, USA) was added for one h, followed by the addition of TGF-β1. pRL-TK renilla luciferase (Promega, Tokyo, Japan) was co-transfected as a control reporter vector. Cell extracts were prepared and subjected to a dual luciferase reporter assay (Promega) as described by the manufacturer.

Western blot analysis

The activation/phosphorylation of p38 MAPK and activating transcription factor (ATF)-2 was determined by Western blotting, as described previously (13). Fibroblasts were grown to visual confluence, and medium was changed to DMEM containing 0.1% bovine serum albumin (BSA). Twenty-four hours later, cells were treated with 5 ng/ml of TGF-β1 (R&D Systems) for various time intervals, and then lysed in Laemmli sample buffer. The sonicated samples were fractionated on 10% sodium dodecyl sulphate-polyacrylamide gels, transferred to polyvinylidene difluoride membranes (Millipore, Bradford, MA, USA), and the membranes were blocked with 5% non-fat milk. Membranes were incubated overnight at 4°C with primary antibodies against phospho-p38 MAPK and phospho-ATF-2 (Cell Signaling Technology, Beverly, MA, USA) diluted 1:1000. After washing, membranes were incubated with appropriate secondary antibody. Specific binding of antibody was visualized by enhanced chemiluminescence detection (Amersham Pharmacia Biotech, Piscataway, NJ, USA). The same blots were stripped and re-probed using antibodies against total p38 MAPK and total ATF-2 (Cell Signaling Technology) diluted 1:1000. Western analysis for β-catenin was performed as described by Cheon et al. (6) with a specific antibody against β-catenin (Cell Signaling Technology) diluted 1:500. As a loading control, membranes were subjected to re-blotting using an antibody against β-actin (Sigma) diluted 1:5000.

Immunoprecipitation

Confluent fibroblasts were incubated in DMEM containing 0.1% BSA for 24 h, followed by stimulation with TGF-β1 (5 ng/ml) for one h. Cells were solubilized in lysis buffer containing 50 mmol/l Tris-HCl, pH 7.4, 150 mmol/L NaCl, 2 mmol/l EDTA, 1% Nonidet P-40, 0.1% SDS, 50 mmol/l sodium fluoride, and 1 mmol/l phenylmethylsulphonyl fluoride. Cell lysates were subjected to immunoprecipitation with the anti-Smad2/3 antibody (N-19, Santa Cruz Biotechnology, Santa Cruz, CA, USA) at 20 μg/ml followed by adsorption to protein G sepharose (Amersham Pharmacia Biotech). Western blotting was performed using an anti-phosphoserine antibody (Zymed, South San Francisco, CA, USA) diluted 1:200. As a loading control, membranes were re-probed with an antibody against Smad3 that reacts with Smad3 and Smad2 (FL-425, Santa Cruz Biotechnology) diluted 1:200.

Immunohistochemistry

Two keloid samples and one hypertrophic scar sample were obtained from different Japanese patients who underwent plastic surgery. As controls, three samples of atrophic scar around skin ulcers were obtained from biopsies and one sample of post-operational atrophic scar was obtained by surgical excision. All subjects provided informed consent. The Institutional Review Board, Dokkyo University School of Medicine, approved the study conducted by the Department of Dermatology (Tochigi, Japan). Diagnoses were confirmed by routine pathological examination. All samples were fixed in buffered formalin and embedded in paraffin following standard protocols. Samples were subjected to immunohistochemical probing for β-catenin as described elsewhere (14). Briefly, tissues were dewaxed and the endogenous peroxidase was quenched with hydrogen peroxide. Heat-induced antigen retrieval was performed using a microwave oven and 10 mmol/l citrate buffer (pH 6) for 15 min. The slides were cooled to room temperature and washed with phosphate-buffered saline. The treated sections were blocked with 1% BSA, and incubated with a monoclonal antibody against β-catenin (Transduction Laboratories, Lexington, KY, USA) at 10 μg/ml overnight at 4°C. After washing, tissues were immunostained using the streptavidin-biotin peroxidase complex method (LSAB universal kit, Dako, Kyoto, Japan).

Immunoreactivity with TGF-β was determined as described previously (15). Formalin fixed and paraffin-embedded samples were dewaxed and pre-treated with 1 mg/ml hyaluronidase buffered with 0.1 mol/l sodium acetate in 1.7 mol/l sodium chloride (pH 5.4). After blocking with 10% normal rabbit serum, slides were incubated with a monoclonal antibody against TGF-β (Genzyme, Cambridge, MA, USA) at 0.15 mg/ml for 30 min. Colour development was performed using an anti-alkaline phosphatase staining kit (Dako).

Statistical analysis

The data were expressed as the mean ± standard deviation (SD) of multiple experiments. Statistical analysis of the data was performed by one-way analysis of variance (ANOVA) followed by Turkey-Kramer multiple comparisons testing.

RESULTS

β-catenin-TCF-mediated transcription is activated by TGF-β through Smad3- and p38 MAPK-dependent mechanisms

To investigate whether TGF-β conveys signals to induce activation of β-catenin signalling, we examined the effect of TGF-β on β-catenin/TCF-induced transcriptional activity. We used a reporter plasmid (pTOPFLASH) that contains three copies of optimal TCF-binding motif CCTTTGATC upstream of a minimal c-Fos promoter driving luciferase reporter gene (12). Treatment of healthy dermal fibroblasts with TGF-β resulted in a

significant 3.2±0.7 -fold increase in luciferase activity relative to untreated pTOPFLASH transfected cells (Fig. 1a). When a reporter plasmid containing mutated TCF-binding sites (pFOPFLASH) was used, little change in reporter activity was observed.

Smad pathways are known to mediate TGF-β signalling. We therefore attempted to determine whether Smad3 mediates the TGF-β-induced, β-catenin/TCF-dependent transcriptional activation. It has been shown previously that Smad3 mediates TGF-β stimulation of collagen production in dermal fibroblasts (16). Furthermore, Smad3 was demonstrated to bind TCF/LEF transcription factors (17). TCF-dependent transcription in human dermal fibroblasts was increased dramatically (24.6±3.7 -fold induction) following transfection of Smad3 (Fig. 1b).

Although Smad proteins are the direct targets for the TGF-β pathway, TGF-β can also activate p38 MAPK (2, 4). Activation of p38 MAPK by TGF-β in human dermal fibroblasts has been demonstrated (5). Next we investigated whether p38 MAPK is involved in the TGF-β-mediated, β-catenin/TCF-dependent transcriptional activation described above. Transfection of p38α resulted in an increase in luciferase activity (4.8±0.8-fold induction). TGF-β and p38α had a synergistic effect on pTOPFLASH promoter activity (13.8 ± 5.1-fold induction) (Fig. 1c). Furthermore, treatment of cells with SB203580, a specific inhibitor of p38 MAPK, led to a decrease in luciferase activity relative to the untreated control (Fig. 1d). These results indicate that both Smad3 and p38 MAPK are likely to be involved in TGF-β-mediated, β-catenin/TCF-dependent transcriptional activation.

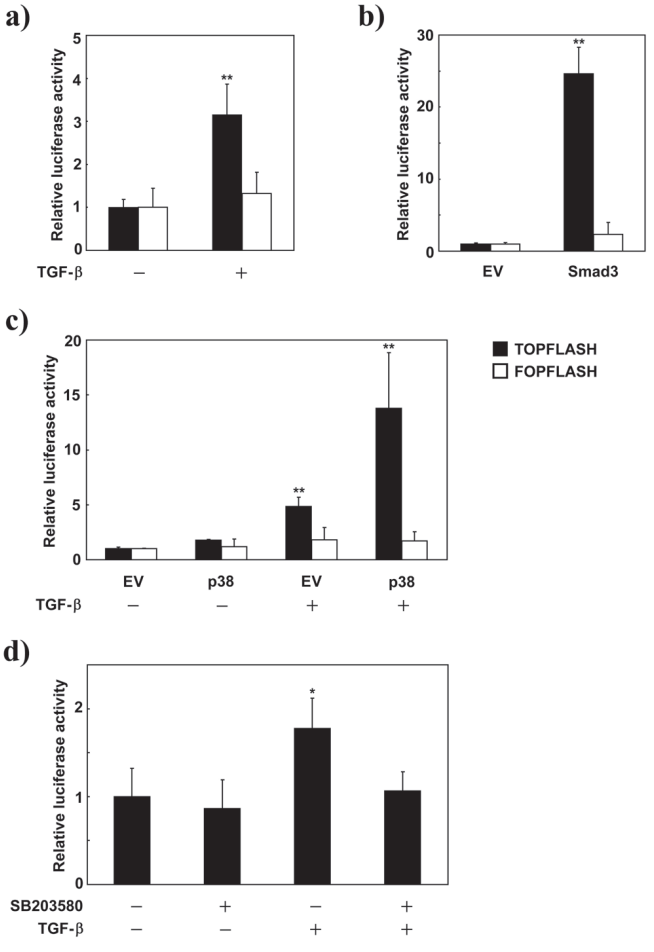

Fig. 1. (a) Effects of TGF-β on β-catenin/TCF-mediated transcription. Normal human dermal fibroblasts were transfected with the TCF-luciferase reporter construct, pTOPFLASH, or the pFOPFLASH control reporter construct containing mutated TCF consensus binding sites. Cells were stimulated with 5 ng/ml TGF-β1 for 24 h and luciferase activity was measured. (b) Effects of Smad3 on β-catenin/TCF-mediated transcription. Normal dermal fibroblasts were transfected with the TCF-luciferase reporter construct, pTOPFLASH, or pFOPFLASH along with either Smad3 expression vector (Smad3) or empty vector (EV). (c) Effects of p38 MAPK on β-catenin/TCF-mediated transcription. Normal dermal fibroblasts were transfected with the TCF-luciferase reporter construct, pTOPFLASH, or pFOPFLASH along with either p38α expression vector (p38) or empty vector (EV). Luciferase activity was determined following treatment with TGF-β1 (5 ng/ml) for 24 h. (d) Effects of SB203580, a specific inhibitor of p38 MAPK, on β-catenin/TCF-mediated transcription. Normal dermal fibroblasts were transfected with the TCF-luciferase reporter construct, pTOPFLASH. Ten μmol/L SB203580 was added for one h, followed by the addition of TGF-β1. Luciferase activity was measured after an additional 24 h. Values represent the mean ± SD of three independent experiments done in duplicate. *$p < 0.05$ compared with control cells. **$p < 0.01$ compared with control cells.

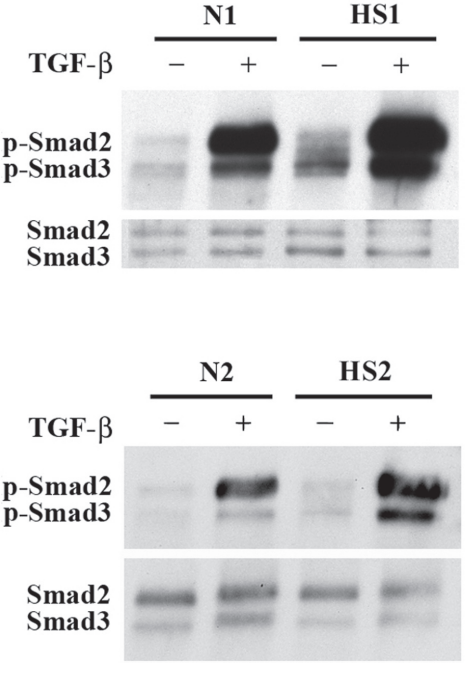

Fig. 2. Analysis of TGF-β induced Smad2/3 phosphorylation in hypertrophic scar (HS) and healthy control (N) fibroblasts. Confluent cells deprived of serum for 24 h were stimulated with 5 ng/ml TGF-β1 for one h. Smad proteins were immunoprecipitated from whole cell lysates with antibody against smad2/3, and immunoblotted with anti-phophoserine antibody. The membrane was re-blotted with anti-Smad3 antibody, which reacts with Smad3 and Smad2. Representative blots from 2 pairs of normal (N1 and N2) and hypertrophic scar (HS1 and HS2) fibroblast cultures are shown.

Increased TGF-β-induced activation of Smad3 in hypertrophic scar fibroblasts

Upon TGF-β stimulation, cellular Smad2 and Smad3 become rapidly phosphorylated by the activated TGF-β receptor serine/threonine kinases. The phosphorylation state of endogenous Smad2/3 thus serves as a marker for the activation of the TGF-β/Smad pathway. We therefore sought to determine the relative phosphorylation state of receptor-activated Smads in hypertrophic scar fibroblasts. We examined TGF-β activation of Smad2/3 in pairs of hypertrophic scar and healthy skin fibroblasts.

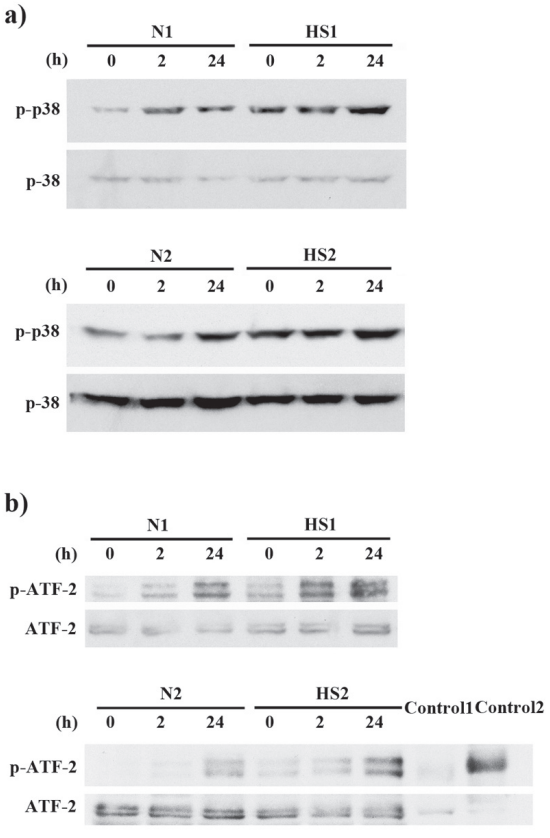

Fig. 3. Western blotting showing time dependent phosphorylation of p38 MAPK and ATF-2 after treatment with TGF-β in hypertrophic scar (HS) and healthy control (N) fibroblasts deprived of serum for 24 h were incubated with TGF-β1 (5 ng/ml) for increasing periods of time as indicated. (a) The levels of phosphorylated p38 (p-p38) MAPK were determined using anti-phospho-p38 MAPK antibody. Total cellular p38 MAPK protein levels are shown as a control. Both cell types demonstrate prolonged activation of p38 MAPK up to 24 h in response to TGF-β. Increased basal levels of p-p38 MAPK are observed in hypertrophic scar fibroblasts (HS1 and HS2) as compared to healthy controls (N1 and N2). (b) The levels of activated ATF-2 (p-ATF-2) and total cellular ATF-2 protein were determined using anti-phospho-ATF-2 and anti-ATF-2 antibody, respectively. Total cell extracts from NIH3T3 cells (control 1) and total cell extracts from anisomycine-treated NIH3T3 cells (control 2) were used as controls. Both cell types demonstrate prolonged activation of ATF-2 up to 24 h in response to TGF-β. Increased basal levels of p-ATF-2 are observed in hypertropic scar fibroblasts (HS1 and HS2) as compared to healthy controls (N1 and N2).

To determine the kinetics of phosphorylation of Smad2/3, fibroblasts were treated with TGF-β (5 ng/ml) for one h. It has been shown in human dermal fibroblasts that TGF-β induces transient phosphorylation of Smad2/3, peaking at one h (18). Representative immunoblots are shown in Fig. 2. Without stimulation of TGF-β, phosphorylation levels of Smad2/3 were hardly detectable in normal fibroblasts. On the other hand, substantial phosphorylation levels of Smad2/3 were detected in one hypertrophic scar cell line (SH1) without treatment with TGF-β. Upon TGF-β stimulation, phosphorylation levels of Smad2/3 were increased in both normal and hypertrophic scar fibroblasts. Both hypertrophic scar fibroblasts displayed slightly elevated Smad3 phosphorylation compared with control fibroblasts after the stimulation with TGF-β.

Increased TGF-β-induced activation of p38 and ATF-2 in hypertrophic scar fibroblasts

Our previous study showed that TGF-β induces activation of p38 MAPK activation in human dermal fibroblasts, contributing to TGF-β induction of the type I collagen gene (5). We examined TGF-β activation of p38 MAPK in two pairs of hypertrophic scar and healthy skin fibroblasts. The total amounts of p38 MAPK were similar in hypertrophic scar and control healthy fibroblasts, and both cell types demonstrated prolonged activation of p38 MAPK up to 24 h. Basal levels of activated p38 MAPK were increased in cell lines of hypertrophic scar fibroblasts compared with healthy skin fibroblasts (Fig. 3a). Since autocrine TGF-β signalling may be responsible for the abnormal phenotype of hypertrophic scar fibroblasts (19), elevated basal levels of p38 MAPK would be expected if this pathway was responsible for constitutive up-regulation of collagen by TGF-β in these cells.

We also examined the phosphorylation levels of ATF-2 induced by the addition of TGF-β, since ATF-2 has been reported to be a common nuclear target of p38

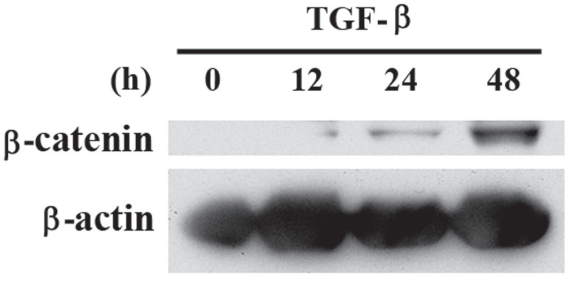

Fig. 4. TGF-β-mediated β-catenin stabilization in human dermal fibroblasts. Confluent cells deprived of serum for 24 h were incubated with TGF-β1 (5 ng/ml) for increasing periods of time as indicated. The total cellular β-catenin protein levels were determined by Western blotting using anti-β-catenin antibody. Equivalent protein loading was demonstrated by re-probing the blot with a monoclonal antibody against β-actin.

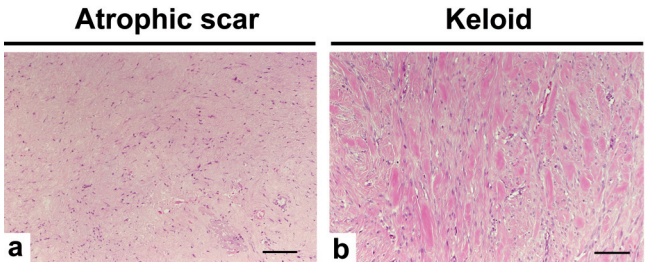

Fig. 5. Histopathology of atrophic scar (a) and keloid (b). Scale bar: 100 μm.

MAPK pathways in TGF-β signalling (20). Western blot analyses revealed elevated ATF-2 phosphorylation levels in two separate cell lines of hypertrophic scar fibroblasts compared with healthy control fibroblasts (Fig. 3b).

β-*catenin protein expression is increased in keloid tissue*

We sought to determine whether TGF-β induces β-catenin accumulation in human dermal fibroblasts. β-catenin protein levels in dermal fibroblasts were barely detectable on Western blots. Upon stimulation with TGF-β, a time-dependent increase in β-catenin protein levels was observed (Fig. 4). This result is consistent with pTOPFLASH activation by TGF-β. Next, we compared the expression levels of β-catenin in response to TGF-β in normal and hypertrophic scar fibroblasts, but did not observe a substantial difference between them (data not shown).

We then asked whether the protein levels of β-catenin are elevated in hypertrophic scar and keloid tissue. We performed immunostaining with anti-β-catenin and compared the immunoreactivity between atrophic scar and keloid or hypertrophic scar samples. Their histological features were identified by routine haematoxylin and eosin staining (Fig. 5). It was recently demonstrated that β-catenin expression is elevated in the proliferation phase of wound samples of mice. In humans, normal liver fibroblasts and lung fibroblasts are reported to be negative for β-catenin (13, 1). We found β-catenin expression in normal skin tissue confined to epidermal keratinocytes, hair follicles, sweat glands and vascular endothelial cells (Fig. 6). Focal immunoreactivity was found in some fibroblasts in the dermal papillae, but no immunoreactivity was found in fibroblasts in the reticular dermis (data not shown). These findings are consistent with other immunohistochemical studies carried out in human skin (22). β-catenin showed intense and diffuse staining in fibroblasts in keloid samples (Fig. 6b). β-catenin immunoreactivity was observed in the cytoplasm, while nuclear accumulation was also noted

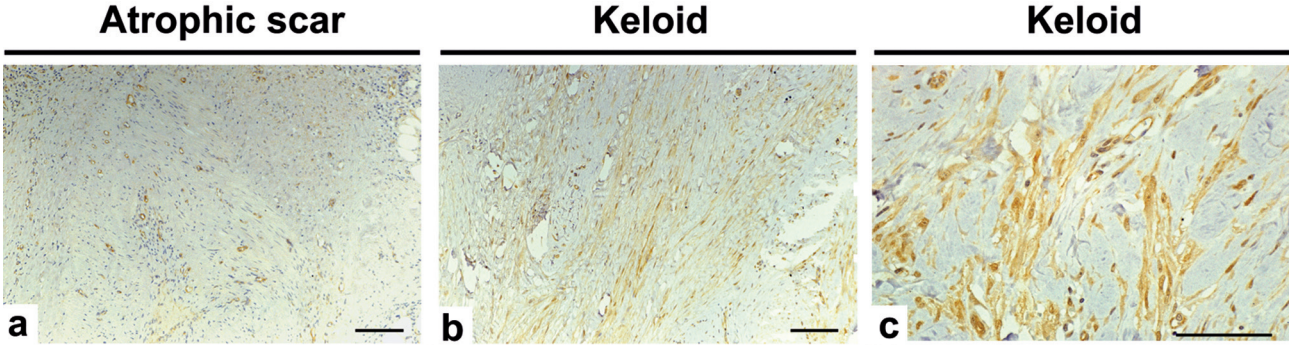

Fig. 6. Immunohistochemistry for β-catenin. (a) Atrophic scar: lack of β-catenin immunoreactivity in dermal fibroblasts. Blood vessels show staining with anti-β-catenin. (b) Keloid: over-expression of β-catenin in fibroblasts. (c) Keloid: β-catenin accumulates in the nucleus as well as in the cytoplasm. Scale bar: 100 μm.

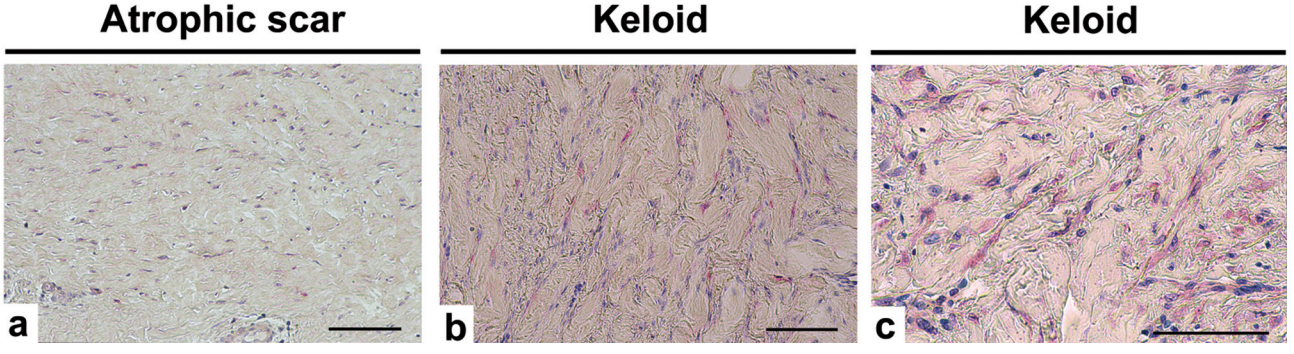

Fig. 7. Immunohistochemistry for TGF-β. (a) Atrophic scar: TGF-β is expressed in a few fibroblasts. (b) Keloid: over-expression of TGF-β in fibroblasts. (c) Keloid: high-power view showing intense staining with anti-TGF-β in fibroblasts. Scale bar: 100 μm.

(Fig. 6c). β-catenin expression was not observed in the normal-appearing spindle cells around the lesions. No immunoreactivity was found in fibroblasts in atrophic scar samples (Fig. 6a). We also examined whether TGF-β is expressed in hypertrophic scar and keloid tissue. As shown previously, normal skin showed moderate staining with TGF-β in the epidermis and appendages, but no staining was observed in the dermis (14). As expected, the immunohistological studies demonstrated TGF-β was over-expressed in fibroblasts in hypertrophic scar and keloid tissue (Fig. 7b and c), whereas low levels of expression were observed in atrophic scar tissue (Fig. 7a).

DISCUSSION

In this study, we showed that the β-catenin/TCF pathway is upregulated by TGF-β through both the Smad3 and p38 MAPK pathways in human dermal fibroblasts. Furthermore, we show that β-catenin accumulates in hypertrophic scar and keloid tissue, suggesting an important role for Wnt signalling in hypertrophic/keloid scarring. Recent evidence has suggested a role for β-catenin in hyperplastic cutaneous wound healing (6, 7). TGF-β stimulation has been demonstrated to induce an elevation of β-catenin protein levels (7, 23). Consistent with these previous studies, the data obtained in this study support the role in wound healing for TGF-β as a positive regulator of Wnt signalling in human dermal fibroblasts. The immunohistochemical studies demonstrate accumulation of β-catenin in hypertrophic scar and keloid tissue, whereas Western blots failed to show an increased accumulation of β-catenin in response to TGF-β in cultured hypertrophic scar fibroblasts (data not shown). Cell behaviour *in vitro* may not necessarily represent the *in vivo* situation. Other cells as well as fibroblasts may be involved in the accumulation of β-catenin in hypertrophic scar and keloid tissue. It is possible that factors other than TGF-β are involved in the Wnt signalling cascade leading to β-catenin accumulation. Further studies are needed to delineate this specific mechanism.

At present, molecular mechanisms for this regulation are not known. It has been shown that nuclear β-catenin influences transcriptional activation by binding to the TCF transcriptional machinery, including a family of high-mobility group factors. The balance between activation and repression of TCF is mediated by competition between co-repressors and TATA box-binding proteins (24). The co-operation between LEF-1 and src through an AP-1 motif via a complex between the LEF-1 and c-Jun transcriptional factors was demonstrated on the matrilysin promoter (25). Labbé et al. (17) showed that Smad3 physically bind and modulate the activity of TCF/LEF transcription factors on certain promoters. Nishita et al. (9) found that Smad4, not Smad3, can bind LEF. In human dermal fibroblasts, the association between TGF-β and β-catenin/TCF signalling pathways may modulate the expression of matrix metalloproteases, collagen, and other connective tissue macromolecules. Further analyses are needed to elucidate the mechanisms of co-operation between the TGF-β and Wnt/β-catenin pathways in the induction of collagen accumulation in cutaneous fibrosis.

We show here that Smad3 is involved in TGF-β activated Wnt/β-catenin signalling. Many studies have revealed that Smad pathways are activated in the wound healing process, and play a part in skin fibrosis of scleroderma (26). Smad3 has been shown to mediate TGF-β-stimulated collagen production (16) and auto-induction of TGF-β (27). Both factors contribute to the development of hypertrophic scars and keloids. As indicated in Fig. 2, low levels of Smad2/3 phosphorylation were detected in the absence of TGF-β in normal fibroblasts. In contrast, substantial endogenous Smad2/3 phosphorylation was observed in one hypertrophic scar cell line (SH1). Smad2/3 phosphorylation levels for SH2 were slightly increased compared with N2 control fibroblasts, while the difference appeared to be small. These results indicate a constitutively activated auto-induction of TGF-β by hypertrophic scar fibroblasts. Hypertrophic scar fibroblasts displayed slightly elevated Smad3 phosphorylation compared with control fibroblasts. Smad3 is known to be a major signal transducer in fibrogenesis (16, 27, 28). Previous studies have demonstrated that TGF-β receptors (types I and II) are increased in keloid fibroblasts (19). More recent studies have demonstrated that an increased TGF-β receptor I/TGF-β receptor II ratio could promote fibrosis (29). Taken together, these studies show that Smad3 phosphorylation by TGF-β plays a critical role in hypertrophic scar and keloid formation.

Besides Smads, TGF-β can activate ERK, JNK and p38 MAPK (4). Our previous study showed that TGF-β induces p38 MAPK activation in human dermal fibroblasts (4). This observation indicates the role of p38 MAPK as a positive regulator of collagen synthesis in dermal fibroblasts and a mediator of TGF-β-stimulated ECM production. However, we failed to demonstrate p38 MAPK involvement in collagen deposition by scleroderma fibroblasts (4). The findings in this study appear to imply that both Smad3 and p38 MAPK contribute to hypertrophic scar and keloid development. Others have speculated that p38 MAPK/ATF-2 is the predominant signalling pathway in keloid formation in the context of TGF-β and insulin-like growth factor-I crosstalk (30). The involvement of p38 MAPK may be the key factor in the causation of hypertrophic/keloid scarring. Functional characterization of both pathways in TGF-β-induced scar formation remains to be done.

Finally, epithelial-mesenchymal transition (EMT) may be involved in the pathogenesis of hypertrophic

scars and keloids. Increasing evidence suggests that Wnt signalling, through its effector β-catenin, is the key regulator of fibroproliferation. Recently, direct evidence has been provided for a role of β-catenin in the induction of EMT. EMT is well-studied in the context of embryonic development and tumour progression. Various cytokines facilitate EMT. Among them, TGF-β is considered to be a prototypical inducer of EMT. EMT has recently been suggested to play an important role in the development of fibrotic diseases (31). There is evidence that EMT is associated with the fibrosis of organs such as kidney and lung (20, 32). In this study, we found β-catenin immunoreactivity in fibroblasts in dermal papillae, consistent with the observations of other investigators (21). We demonstrated that β-catenin protein levels are elevated in hypertrophic scar and keloid tissue, but not in atrophic scar tissue. These findings may be related to the possible involvement of EMT in the development of hyperplastic cutaneous fibrosis.

In conclusion, we have described a novel role for β-catenin in keloid formation. That is, the TGF-β signalling pathway works in a combinatorial manner with Wnt/β-catenin signalling and the co-operation between TGF-β and β-catenin signalling pathways might be relevant to the pathogenesis of hypertrophic scars and keloids. However, the data shown here is based on a relatively small number of samples. Additional samples and experiments are necessary further to confirm this conclusion.

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