Insulin-Like Growth Factor-I Enhances Transforming Growth Factor-β-Induced Extracellular Matrix Protein Production Through the P38/Activating Transcription Factor-2 Signaling Pathway in Keloid Fibroblasts

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Keloids are benign dermal tumors, characterized by invasive growth of fibroblasts and concomitant increased biosynthesis of extracellular matrix components, with unclear etiology. We previously demonstrated that keloid fibroblasts overexpress insulin-like growth factor-I receptor. In investigating the role of insulin-like growth factor-I receptor overexpression, insulin-like growth factor-I and transforming growth factor-β interaction was examined in relation to extracellular matrix protein production in cultured human and mouse fibroblasts. Western blotting revealed that collagen type I was expressed in keloid and normal fibroblasts, and its expression was increased by transforming growth factor-β stimulation more significantly in keloid rather than in normal fibroblasts. Insulin-like growth factor-I and transforming growth factor-β1 costimulation markedly increased extracellular matrix proteins (collagen type I, fibronectin, and plasminogen activator inhibitor-1) compared with cultures with transforming growth factor-β1 alone. Insulin-like growth factor-I treatment alone had no stimulatory effect. Real-time reverse transcription–polymerase chain reaction confirmed parallel collagen type I messenger RNA level changes. Luciferase assays were conducted to investigate intracellular signaling pathways in this synergistic stimulation using a mouse fibroblast cell line. Transforming growth factor-β1 (1 or 10 ng per ml) increased the specific signaling activity approximately 10-fold, whereas the increase with insulin-like growth factor-I (100 ng per ml) was less than 2-fold compared with basal activity; however, the combination of transforming growth factor-β1 and insulin-like growth factor-I resulted in an approximately 25-fold increase. Insulin-like growth factor-I markedly enhanced transforming growth factor-β-induced phosphorylation of p38 mitogen-activated protein kinase and activating transcription factor-2. Luciferase assay showed that this synergistic effect was attenuated by the p38 mitogen-activated protein kinase specific inhibitor SB203580 or phosphatidylinositol 3-kinase inhibitor wortmannin, but not by the mitogen-activated protein kinase/extracellular-signal-regulated protein kinase kinase inhibitor PD98059. These results indicate that insulin-like growth factor-I enhances transforming growth factor-β-induced keloid formation through transforming growth factor-β postreceptor signal cross-talk, mainly via the p38 mitogen-activated protein kinase/activating transcription factor-2 pathway. ***Key words: fibrosis/mitogen-activated protein kinase/plasminogen activator inhibitor-1/collagen type I/fibronectin. J Invest Dermatol 120:956–962, 2003***

Keloids are classified as benign dermal tumors, characterized by the proliferation of dermal fibroblasts, overproduction of extracellular matrix components (ECM), invasiveness beyond the original boundary of the insult, and recurrence. Transforming growth factor (TGF)- β1 has been implicated in several fibrotic disorders, including glomerulonephritis, liver cirrhosis, and lung fibrosis (Border and Noble, 1994). In dermal fibroblasts, TGF-β1 is known to play a crucial part in wound healing processes. TGF-β1 has also been shown to markedly enhance the expression of ECM proteins by cultured fibroblasts, and it has therefore been postulated that TGF-β1 plays a significant part in the development of keloids (Babu *et al*, 1992; Younai *et al*, 1994; Tuan and Nichter, 1998; Chin *et al*, 2001). Furthermore, TGF-β1 is involved in the anti-apoptotic property of keloid fibroblasts (Chodon *et al*, 2000). TGF-β1 mediates signaling through two transmembrane serine/threonine kinase receptors, type I and type II TGF-β receptors. The constitutively active type II receptor recruits the type I receptor upon ligand binding and then phosphorylates its serine and threonine residues in a glycine serine rich domain. Once phosphorylated, the type I receptor activates downstream targets.

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Abbreviations: IGF-I, insulin-like growth factor-I; ECM, extracellular matrix; PAI-1, Plasminogen activator inhibitor-1; MAPK, mitogen-activated protein kinase; MEK, MAPK/extracellular-signal-regulated protein kinase kinase; ATF-2, activating transcription factor-2; PI3K, phosphatidylinositol 3-kinases; JNK, Jun N-terminal kinase.

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Recent evidence indicates that TGF-β1 transduces signals through two different pathways, Smad and mitogen-activated protein kinase (MAPK), including p38, Jun N-terminal kinase (JNK), and extracellular signal regulated kinase (ERK) (Zhou *et al*, 1998; Hanafusa *et al*, 1999; Sato *et al*, 2002).

Insulin-like growth factor-I receptor (IGF-IR) is composed of two extracellular α-subunits and two transmembrane β-subunits that possess intrinsic tyrosine kinase activity (Ullrich and Schlessinger, 1990). Activated IGF-IR phosphorylates various substrates on tyrosine residues, including those of the Ras-Raf-MAPK pathway (Rozakis-Adcock *et al*, 1993; Skolnik *et al*, 1993) and the phosphatidylinositol 3-kinase (PI3K) pathway (Backer *et al*, 1992). In previous studies, we found that keloid fibroblasts overexpressed IGF-IR, IGF-I mediated invasiveness of keloid fibroblasts (Yoshimoto *et al*, 1999; Ohtsuru *et al*, 2000), and keloid fibroblasts are resistant to apoptosis due to IGF-I signaling (Ishihara *et al*, 2000). Although IGF-I/IGF-IR signaling is involved in keloid formation, it is not clear whether IGF-I can modulate TGF-β-mediated effect. In this study we examined how IGF-I affects TGF-β-induced ECM production, focusing on the postreceptor signaling pathway of TGF-β action.

MATERIALS AND METHODS

Materials Keloid samples were obtained from three different Japanese patients after surgical excision and the diagnosis confirmed by routine pathologic examination. In all cases, keloids had developed at sites of unsuspected injury (trivial wounds and acne) and had not been subjected to any kind of treatment. Three normal skin samples were obtained from three Japanese volunteers. Informed consent was obtained from each individual and the study approved by the ethical committee of Nagasaki University (no. 13020615). Patient profiles are listed in **Table I**.

Cell cultures Primary cultures of dermal fibroblasts were established as previously described (Ishihara *et al*, 2000). Explants were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (Invitrogen, Burlington, Ontario, Canada), 100 U per ml penicillin, and 100 μg streptomycin per ml at 37°C in a humidified incubator with 5% CO_2. Fibroblasts were treated with TGF-β1 (Invitrogen), IGF-I (Sigma, St Louis, MO) or both, at passages 3–8. A hepatoma cell line, HepG2, was cultured as previously described (Akiyama-Uchida *et al*, 2002).

Western blot analysis As the ECM proteins such as collagen type I, fibronectin, and plasminogen activator inhibitor (PAI)-1 are produced and then secreted into extracellular space, we used culture media but not cell lysates as loading samples after the measurement of protein concentration using Bio-Rad assay (BIO-RAD, Richmond, CA). After stimulation for 12 h, culture media was treated with sodium dodecyl sulfate lysis buffer (62.5 mM Tris–HCl, pH 6.8, 2% sodium dodecyl sulfate, 10% glycerol, 50 mM dithiothreitol, and 0.1% bromophenol blue). The proteins were resolved in 7.5% sodium dodecyl sulfate polyacrylamide gels and transferred to nitrocellulose membranes (Hybond-P; Amersham, Arlington Height, IL). The membranes were preincubated with a blocking buffer for 1 h at room temperature. They were then incubated with the primary antibodies to collagen type I (Rockland Immunochemicals, Gilbertsville, PA), fibronectin (Calbiochem, San Diego, CA), PAI-1 (American Diagnostics Inc, Greenwich, CT), phospho-activating transcription factor (phospho-ATF)-2 and control-ATF-2 (Cell Signaling Technology, Beverly, MA), p38 MAPK (New England Biolabs, Hercules, CA), and JNK antibody (Santa Cruz Biotechnology, Santa Cruz, CA). This was followed by incubation with the secondary antibody, a horseradish peroxidase-conjugated goat anti-rabbit IgG (Santa Cruz Biotechnology). Proteins were visualized with a chemiluminescent detection system as proscribed by the manufacturer (ECL; Amersham).

Table I. Characteristics of individuals included in the study

	(age, years)	(sex)	(biopsy site)	(duration)*
Keloid				
K–1	77	female	Chest	10 years
K–2	36	female	Shoulder	3 years
K–3	64	male	Chest	45 years
Normal				
N–1	6	male	Foot**	
N–2	43	female	Back	
N–3	17	male	Thigh	

Controls are not site matched.
*Keloid persistence befor the surgery
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Expression of collagen type I mRNA Total RNA was extracted from each sample stimulated in various ways for 6 h. This time point was determined as optimal by the time course study by Luciferase assay (data not shown). One microgram of total RNA reverse transcribed for 1 h at 41°C using random hexamers in a 25 μl reaction. Then 1 μl was used as a template for real-time polymerase chain reaction (PCR), which was carried out in an ABI PRISM® 7700 Sequence Detection System (PE Applied Biosystems, Warrington, U.K.) using the SYBR Green kit (PE Applied Biosystems) according to the manufacturer's guidelines. For each sample, the relative mRNA level of collagen type I was expressed in arbitrary units after normalization against α-tubulin. Standard curves for each real-time PCR run were generated from serial dilutions of a reverse transcription reaction with high content of both collagen I and tubulin mRNA. For each type of real-time PCR assay, 40 biphasic cycles of denaturation at 95°C for 15 s followed by annealing/extension at 60°C for 1 min were performed. Samples were analyzed in triplicates. The primer sequences were as follows: collagen type I (COL1A1) sense ACGCACGGCCAAGAGGAA and anti-sense CGTTGTCGCAGACGCAGATC; α-tubulin sense AGATCATTGACCTCGTGTTGGA and anti-sense ACCAGTTCCCCCACCAAAG; their concentration in the reaction mixture was 200 nM.

Luciferase assay Plasmid p3TP-Lux contains the luciferase reporter gene, under the control of a portion of the PAI-1 promoter region, and three consecutive phorbol ester 12-O-tetradecanoyl phorbol-13-acetate (TPA) response elements. Human FAST-1 (human forkhead activin signal transducer-1) (hFAST-1) possesses the ability to bind human Smad2 and activates an activin response element (ARE). Thus the ARE-Lux fusion luciferase vector was used for transcriptional activity determination with FAST-1, to detect Smad signals (Akiyama-Uchida *et al*, 2002). pRL-CMV *Renilla* luciferase was cotransfected as a control reporter vector. Cultured normal and keloid fibroblasts and HepG2 cells at 70% confluence were transiently transfected using the Lipofectamine procedure. The media was changed to serum-free Dulbecco's modified Eagle's medium, and the culture continued for 24 h at 37°C. Cells were incubated for a further 12 h with IGF-I or TGF-β1, or a combination of the two. Cell extracts were prepared and subjected to dual-luciferase assay (Promega, Madison, WI) as proscribed by the manufacturer. To investigate the involvement of p38 and the ERK pathway in the induction of 3TP-Lux, we used the specific inhibitor of p38, SB203580 (5 or 10 μM) (Calbiochem), the MEK1 inhibitor, PD98059 (5 or 10 μM) (New England Biolabs. Inc., Beverly, MA) and the PI3K inhibitor, wortmannin (10 or 100 nM) (Wako Chemical, Osaka, Japan).

RESULTS

Expression of ECM proteins on dermal fibroblasts **Figure 1**(***A,B***) shows representative western blots for collagen type I expression in primary cultures of normal and keloid human fibroblasts. Basal expression of collagen type I is observed in keloid and normal fibroblasts. TGF-β1 treatment enhanced its expression in both fibroblast cultures (**Fig 1*A***). IGF-I caused some changes in levels of collagen type I and fibronectin expression, and treatment with TGF-β1 and IGF-I in combination markedly upregulated the expression of both proteins compared with either IGF-I or TGF-β1 treatment alone (**Fig 1*B,C***). We then examined the expression of PAI-1 as a marker of fibrosis through TGF-β1 signaling. As shown in **Fig 1**(***D***), treatment of keloid fibroblasts with TGF-β1 and IGF-I in combination also markedly upregulated PAI-1 expression compared with control, IGF-I, or TGF-β1 treatment alone. Similar results were obtained in normal fibroblasts, but the combination effects were not as strong.

Expression of collagen type I mRNA **Figure 2** shows the expression of collagen type I mRNA in primary cultures of normal and keloid human fibroblasts estimated by real-time

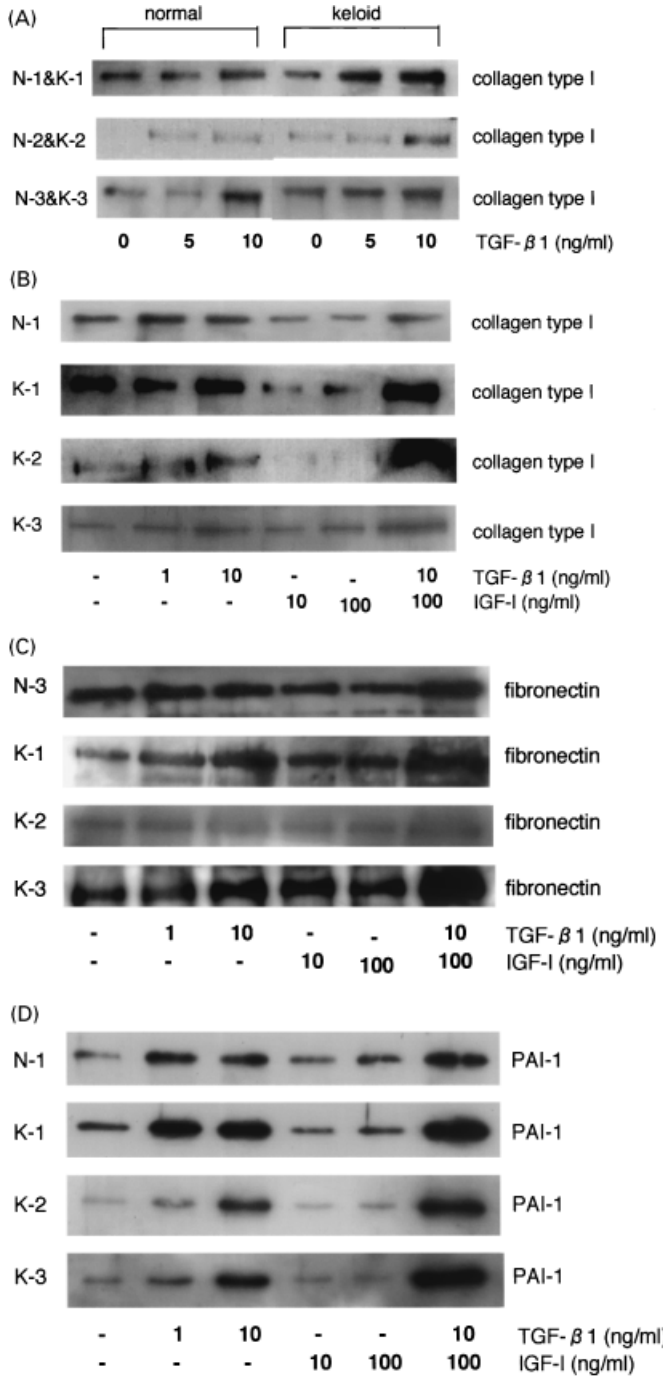

Figure 1. Western blotting of ECM proteins in culture media from dermal fibroblasts. (*A*) Concentration-dependent effect of TGF-β1 stimulation on collagen type I secretion by cultured human fibroblasts. *Lanes 1–3*: normal fibroblasts (N-1–3); *lanes 4–6*: keloid fibroblasts (K-1–3). Equivalent protein load was controlled by Bio-Rad protein assay kit. (*B*) Collagen type I expression after treatment with TGF-β1 (1 or 10 ng per ml) and IGF-I (10 or 100 ng per ml) separately, or in combination in normal (N-1) and keloid fibroblasts (K-1, 2). (*C*) Fibronectin expression after treatment with TGF-β1 (1 or 10 ng per ml), IGF-I (10 or 100 ng per ml), or both in combination in normal (N-3) and keloid fibroblasts (K-2, 3). (*D*) PAI-1 expression after treatment with TGF-β1 (1 or 10 ng per ml), IGF-I (10 or 100 ng per ml), or both in combination in normal (N-1) and keloid fibroblasts (K-1, 3).

reverse transcription–PCR. The combination treatment with TGF-β1 and IGF-I upregulated the expression of collagen type I mRNA in keloid fibroblasts nearly 2.2-fold compared with 1.5-fold in cells treated with TGF-β alone. In normal fibroblasts, this

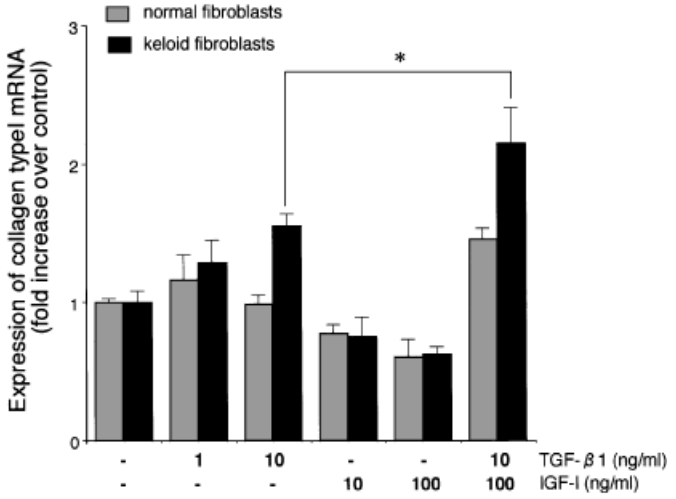

Figure 2. Expression of collagen type I mRNA after treatment with TGF-β1 (1 or 10 ng per ml), IGF-I (10 or 100 ng per ml) separately, or in combination in cultured fibroblasts. RNA was extracted from cultures, reverse transcribed, and analyzed by quantitative reverse transcription–PCR using real-time PCR. Results are expressed as fold increases of collagen I mRNA after normalization to α-tubulin. $^{*}p < 0.05$.

synergistic effect was not significant. IGF-I treatment alone, however, slightly downregulated the expression of collagen type I mRNA in both normal and keloid fibroblasts.

TGF-β signaling in mouse fibroblasts We treated BALB/C 3T3 mouse fibroblasts with TGF-β1 or IGF-I alone or in combination to assess whether Smad or p38 MAPK were acting dominantly downstream of the TGF-β1 receptor. **Figure 3(*A*)** shows that 10 ng IGF-I per ml treatment did not increase 3TP-Lux activity, whereas 100 ng IGF-I per ml treatment resulted in a small increase. TGF-β1 treatment (1 ng per ml or 10 ng per ml) produced an approximately 10-fold increase in activation. At the same time, the combination of the two factors increased 3TP-Lux activity approximately 25 times. 3TP-Lux activity was not increased by the combination treatment compared with TGF-β alone in HepG2 cells, indicating that the synergistic effect of TGF-β1 and IGF-I is cell-type specific.

We then determined whether the Smad cascades of TGF-β1 signaling were activated by IGF-I alone, or TGF-β1 and IGF-I in combination (**Fig 3*B***). TGF-β1 treatment (10 ng per ml) alone increased ARE activity in the presence of FAST-1, but TGF-β1 and IGF-I in combination did not further enhance the activity of ARE-Lux. ARE-Lux activity without FAST-1 cotransfection was unchanged by various combination treatments. In HepG2 cells, ARE-Lux activity was markedly induced by TGF-β1 treatment with FAST-1 cotransfection, but no synergistic effect was produced by TGF-β1/IGF-I combination.

3TP-Lux activity in human fibroblasts To confirm synergistic signal transduction in normal and keloid fibroblasts, we used the 3TP-Lux assay in normal and keloid fibroblasts. As shown in **Fig 4**, treatment with 100 ng per ml IGF-I did not enhance 3TP-Lux activity significantly, whereas 10 ng per ml TGF-β1 produced an increased activation of approximately 3- or 4-fold in normal and keloid fibroblasts, respectively. Furthermore, combination treatment produced a rise in activity of approximately 4-fold in normal fibroblasts, and an 8-fold increase in keloid fibroblasts.

Downstream signaling of TGF-β-induced MAPK cascades in human fibroblasts To examine the effect of intervention at various stages of the MAPK cascade on TGF-β1 and IGF-I signaling in human fibroblasts, we used SB203580 (a p38-specific inhibitor) and PD98059 (a MEK specific inhibitor). As

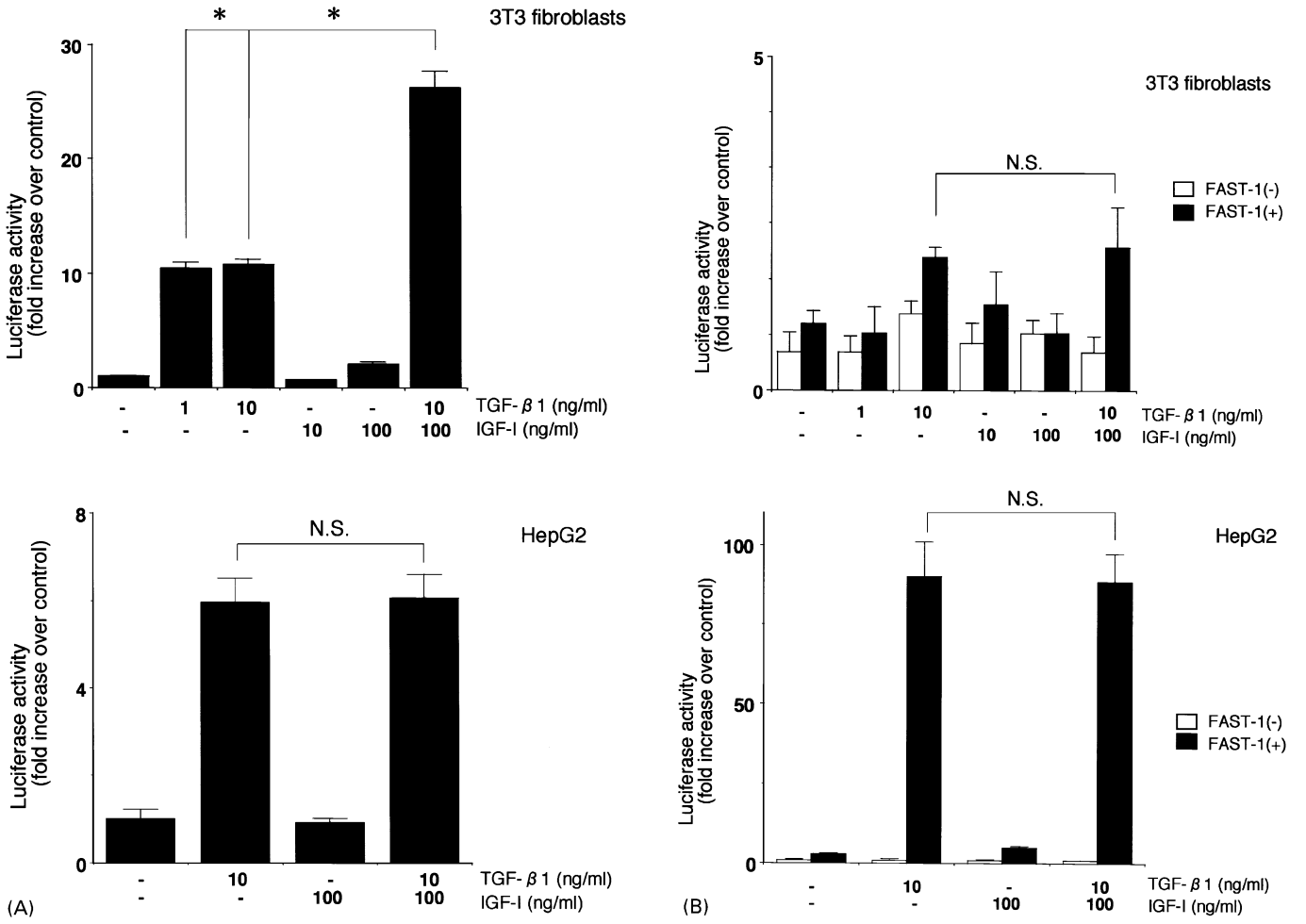

Figure 3. (*A*) 3TP-lux activity in BALB/C 3T3 mouse fibroblasts and HepG2 cells after treatment with TGF-β1 (1 or 10 ng per ml), IGF-I (10 or 100 ng per ml), or with both in combination. 3T3 fibroblasts and HepG2 cells transfected with 3TP-Lux were incubated for 12 h with various stimulants. (*B*) **ARE-lux activity in BALB/C 3T3 mouse fibroblasts and HepG2 cells after treatment with TGF-β1 (1 or 10 ng per ml), IGF-I (10 or 100 ng per ml) separately, or both in combination**. 3T3 fibroblasts and HepG2 cells were cotransfected with ARE-Lux in the presence or absence of FAST-1 before stimulation. Results are expressed as fold increases above the values for untreated controls (mean ± SE, n = 6). *p < 0.03, N.S., nonsignificant.

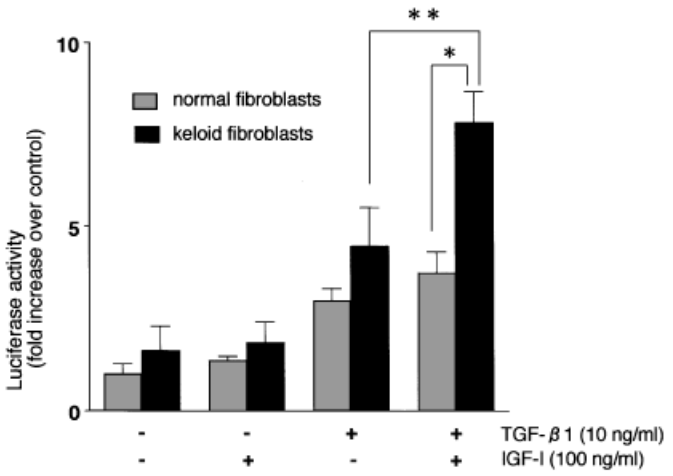

Figure 4. 3TP-lux activity in normal and keloid human fibroblasts treated with TGF-β1 (10 ng per ml), IGF-I (100 ng per ml) separately, or with both in combination. Normal and keloid fibroblasts transfected with 3TP-Lux were incubated 12 h with the stimulants. Results are expressed as fold increases above the values for untreated controls (mean ± SE, n = 6) in normal fibroblasts. *p < 0.01; **p < 0.03.

shown in **Fig 5**, the synergistic effect was almost completely blocked by SB203580, but not by PD98059, in both normal and keloid fibroblasts. Furthermore, to examine whether PI3K correlates with the enhancement of TGF-β signaling, we used wortmannin, a PI3K inhibitor. As shown in **Fig 6**, wortmannin almost abrogated the synergistic effect of IGF-I. We next examined whether phosphorylation of p38 and ATF-2 is enhanced by combination treatment with IGF-I and TGF-β1, as ATF-2 has been reported to be a common nuclear target of p38 MAPK pathways in TGF-β signaling (Gupta *et al*, 1995). TGF-β1 (10 ng per ml) doubled the level of phospho-ATF-2, and treatment with IGF-I and TGF-β1 enhanced it by approximately 3-fold (**Fig 7*A***). In contrast, treatment with IGF-I alone resulted in little change from control levels. Western blot analysis of phospho-p38 MAPK gave similar results: combination treatment markedly increased phosphorylation of p38 compared with either IGF-I or TGF-β1 treatment alone (**Fig 7*B***). Finally, we examined whether JNK activation correlates with synergistic effect of TGF-β and IGF-I; however, the phosphorylation level of JNK was not changed by various treatments (**Fig 7*C***).

DISCUSSION

In this study, the expression of collagen type I and fibronectin revealed that keloid fibroblasts are more responsive to synergistic

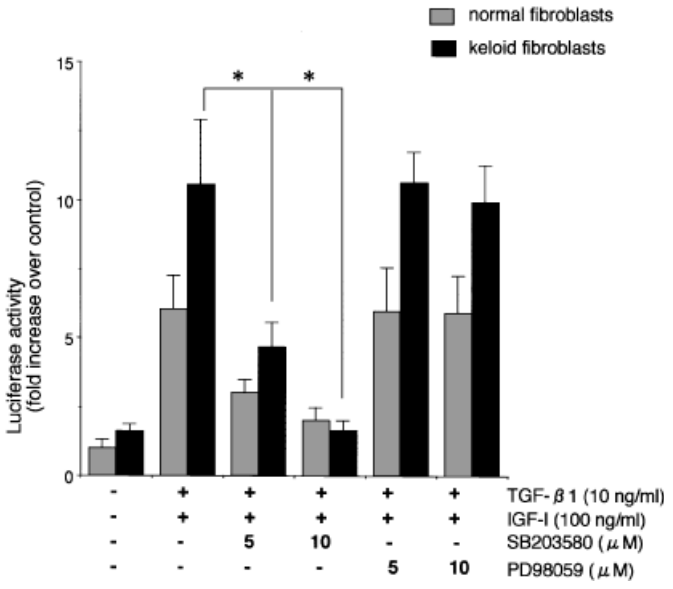

Figure 5. 3TP-lux activity in normal and keloid fibroblasts. The effect on MAPK pathways was examined by pretreatment with either PD98059 or SB203580 before administration of IGF-I (100 ng per ml), TGF-β1 (10 ng per ml) separately, or both in combination. PD98059 is a specific inhibitor of MEK1, and SB203580 is a specific inhibitor of p38. *$p < 0.03$

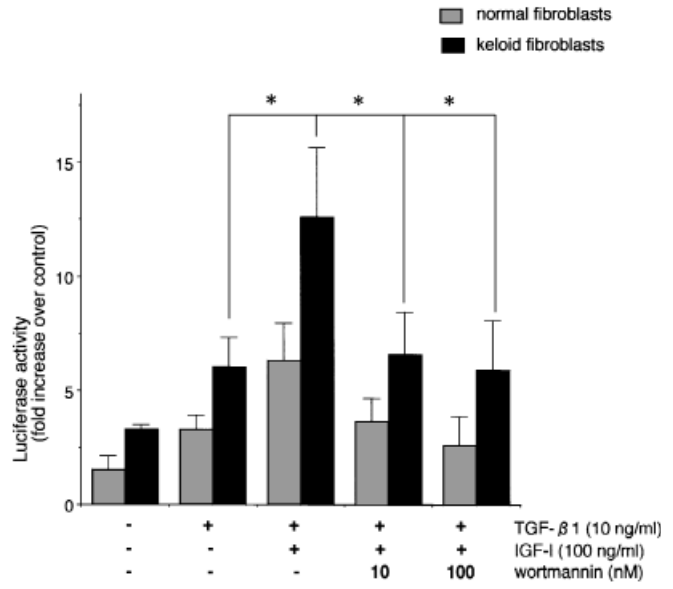

Figure 6. 3TP-lux activity in normal and keloid fibroblasts. The effect on PI3K pathways was examined by pretreatment with wortmannin (10 nM or 100 nM) before administration of IGF-I (100 ng per ml), TGF-β1 (10 ng per ml) or both in combination. Results are expressed as fold increases above the values for untreated controls (mean ± SE, $n = 6$). *$p < 0.03$.

stimulation induced by the combination of TGF-β1 and IGF-I than normal fibroblasts. Even on an mRNA level, real-time reverse transcription–PCR confirmed parallel collagen type I changes. Although the expression of collagen type I was slightly downregulated by IGF-I treatment alone on protein and mRNA levels, the combination of TGF-β and IGF-I enhanced its expression, suggesting that IGF-I plays a modulating role in TGF-β1-stimulated ECM protein secretion. Immunoblot analysis for PAI-1 demonstrated a synergistic effect of IGF-I with TGF-β1, suggesting that fibrosis may be accelerated as a result of suppression of fibrinolysis in keloids. To clarify the signaling mechanisms of this synergism, we employed the TGF-β *trans*-acting reporter system. 3TP-Lux activity was increased approximately 8-fold when keloid fibroblasts were treated with TGF-β1 and IGF-I, but not following treatment with IGF-I alone. Ghahary *et al* (2000) previously reported that IGF-I induces the expression of latent TGF-β1 through activation of c-*fos* and c-*jun* oncogenes; however, our data show that IGF-I stimulation alone did not increase the level of 3TP-Lux activity in time along with ECM protein production in keloid fibroblasts.

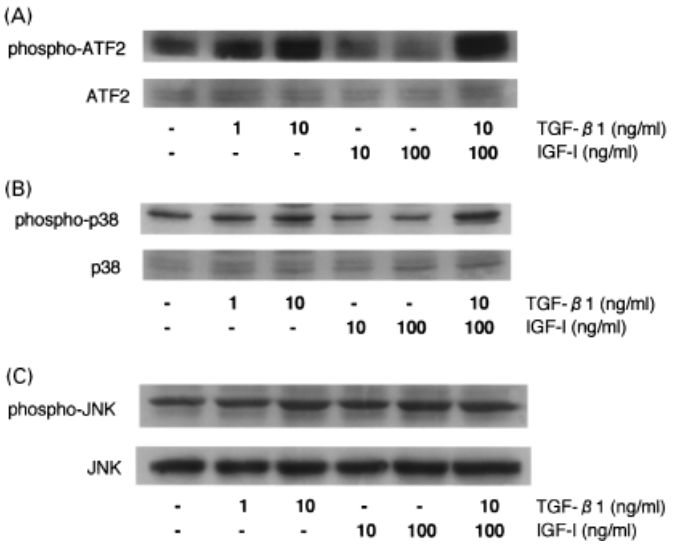

Figure 7. Western blotting showing phosphorylation of ATF-2, p38, and JNK after treatment with TGF-1 (1 or 10 ng per ml), IGF-I (10 or 100 ng per ml) separately, or both in combination in keloid fibroblasts.

Smad are a family of proteins that operate downstream of various members of the TGF-β superfamily (Heldin *et al*, 1997) with Smad2 and Smad3 being downstream effectors of the TGF-β signaling pathway. Upon ligand binding, they are phosphorylated by the TGF-β1 type I receptor kinase and translocate to the nucleus in a complex with Smad4 (Huang *et al*, 1995). This heteromeric complex may either bind directly to the promoters of its target genes, or associate with other transcription factors to induce gene transcription (Derynck *et al*, 1998). Recent work has identified a potential consensus Smad3–Smad4 DNA binding site, GTCTAGAC (Zawel *et al*, 1998), which is observed within the 3TP-Lux promoter and ARE-Lux promoter (Yingling *et al*, 1997; Dennler *et al*, 1998). This suggests that Smad pathways influence the activation of 3TP-Lux as do MAPK pathways. Recent studies revealed that Smad pathways are activated in the wound healing process, and may play some part in fibrosis (Verrecchia and Mauviel, 2002). To elucidate whether the synergistic effect of IGF-I on TGF-β1 signaling is mediated by Smad, we examined the ARE-Lux reporter system, both in the presence and absence of FAST-1. As FAST-1 specifically binds to Smad2, the FAST-1-dependent activation of the ARE-Lux promoter is mediated by endogenous Smad4 (Frey and Mulder, 1997). In this study, the ARE-Lux activity in the presence of FAST-1 increased approximately 80-fold over the control level in HepG2 cells, but only by approximately 2.5-fold in 3T3 fibroblasts, treated with TGF-β1. In addition, IGF-I did not enhance ARE-Lux activity in either HepG2 or 3T3 fibroblasts. Based on these results, it is plausible that MAPK predominantly influence ECM production by cultured fibroblasts treated with TGF-β and IGF-I, but Smad do not; however, Smad3 itself has been demonstrated to be involved in various fibrogenesis models, including keloids (Roberts *et al*, 2001; Chin *et al*, 2001). Further study is required to clarify the role of Smad3 in keloid formation.

Recently, we and other groups have reported that TGF-β1 can activate MAPK, ERK, and p38 protein kinase (Hanafusa *et al*,

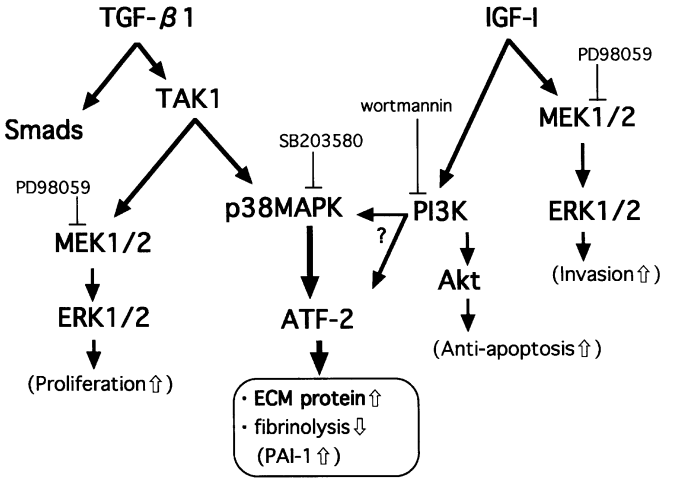

Figure 8. A possible molecular mechanism of keloid formation mediated by TGF-β1 and IGF-I cross-talk.

1999; Finlay *et al*, 2000; Ravanti *et al*, 2001; Akiyama-Uchida *et al* 2002; Sato *et al*, 2002). As TAK1 can activate the p38 MAPK pathway, we examined whether specific inhibitors of this signaling pathway can block 3TP-Lux activity. As shown in **Fig 5**, the synergistic effect of IGF-I and TGF-β1 was almost completely abrogated by the p38 inhibitor, SB203580, but not by the MEK inhibitor, PD98059, suggesting that p38 MAPK pathways are predominantly responsible for the regulation of induction of gene expression by TGF-β1. Alternatively, IGF-I activates MAPK pathways (ERK1/2) and PI3K pathways (Backer *et al*, 1992; Rozakis-Adcock *et al*, 1993; Skolnik *et al*, 1993). MEK inhibitor did not change the 3TP-Lux activity, indicating that the synergistic effect is independent on IGF-I/MEK/ERK pathway. As shown in **Fig 6**, the modulating effect of IGF-I was almost completely blocked by the PI3K specific inhibitor, wortmannin. Therefore, the specific inhibition of TGF-β1 signaling pathways suggests that PI3K pathways could be prevalent in the regulation of ECM production downstream of the IGF-I receptor. ATF-2, a member of the ATF/cyclic adenosine monophosphate response element binding protein (CREB) family of transcription factors, can form dimers through its leucine zipper structure and bind to CRE (Kara *et al*, 1990). p38 phosphorylates ATF-2 at Thr-69, Thr-71, and Ser-90 (Gupta *et al*, 1995). Our western blotting analysis demonstrated that phospho-ATF-2 was markedly increased by treatment with IGF-I and TGF-β1.

Although the precise cause of overexpression of IGF-IR in keloid fibroblasts is as yet unknown, it is highly possible that IGF-I/IGF-IR signaling could lead to characteristic keloid activities, such as proliferation, invasion, anti-apoptosis, and excess ECM production. **Figure 8** shows a proposed molecular mechanism of keloid formation mediated by TGF-β and IGF-I cross-talk. TGF-β stimulates cell proliferation via the ERK pathway (Pena *et al*, 2000). In previous studies, IGF-I has been associated with anti-apoptosis and invasive potential of keloid fibroblasts (Yoshimoto *et al*, 1999; Ishihara *et al* 2000). In this study, we have described a novel mechanism for keloid formation, in particular for the excess ECM production. IGF-I enhances fibrosis during keloid formation through TGF-β1 postreceptor signaling, predominantly via the p38 MAPK/ATF-2 pathway. The exact nature of this complex interaction between TGF-β and IGF-I involving cross-talk comprises a focus for further study on keloid formation.

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