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P38 MAP kinase mediates transforming growth factor-β2 transcription in human keloid fibroblasts

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Xia, Wei, Michael T. Longaker, and George P. Yang. P38 MAP kinase mediates transforming growth factor-β2 transcription in human keloid fibroblasts. *Am J Physiol Regul Integr Comp Physiol* 290: R501–R508, 2006; doi:10.1152/ajpregu.00472.2005.—Keloids are abnormal fibrous growths of the dermis that develop only in response to wounding and represent a form of benign skin tumor. Previous studies have shown increased protein levels of TGF-β in keloid tissue, suggesting a strong association with keloid formation leading us to examine mechanisms for why it is more highly expressed in keloids. Here, we use serum stimulation as an in vitro model to mimic a component of the wound microenvironment and examine differential gene expression in keloid human fibroblasts (KFs) vs. normal human fibroblasts (NFs). Transcription of TGF-β2 was rapid and peaked between 1 and 6 h after serum stimulation in KFs vs. NFs. We confirmed increased TGF-β activity in the conditioned medium from KFs, but not NFs. Inhibition of second messenger signaling pathways demonstrated that only the p38 MAPK inhibitor SB-203580 could block upregulation of TGF-β2 following serum stimulation in KFs. Immunoblotting demonstrated that p38 MAPK was phosphorylated within 15 min and was maintained at a high level in KFs but not in NFs. The transcription factors activating transcription factor-2 and Elk-1 are activated by p38 MAPK, and also showed rapid and prolonged phosphorylation kinetics in KFs but not in NFs. In conclusion, increased TGF-β2 transcription in response to serum stimulation in KFs appears to be mediated by the p38 MAPK pathway. This suggests the mechanism of keloid pathogenesis may be due in part to an inherent difference in how the fibroblasts respond to wounding.

serum stimulation; stress response; keloid pathogenesis

**Keloids are abnormal fibrous proliferations of the dermis that develop only in response to skin injury. The disease is characterized by persistent growth of the wound tissue after reepithelialization and extension of scar tissue beyond the original borders of the wound. Keloids can be very disfiguring, and their presence can cause disability due to contracture and loss of mobility if they are overlying a joint. Treatments, including steroid injections and radiation, have limited efficacy, and surgical excision often results in recurrence (25). We believe the lack of effective therapies is due to an inadequate understanding of the disease pathology. The study of keloid biology is further complicated by the lack of a well-established animal model for the disease.**

**TGF-β is a potent profibrotic factor thought to be involved in keloid pathogenesis. The association of TGF-β with keloid formation has been well documented. Many groups have demonstrated increased levels of TGF-β in keloid tissue (9, 21, 28), as well as elevated sensitivity to TGF-β of keloid human fibroblasts (KFs) (3, 4, 18, 28, 35). TGF-β2 is important in almost every step in the process of tissue fibrosis by simultaneously signaling fibroblasts to increase the synthesis of matrix proteins, decrease the production of matrix-degrading proteases, and increase the production of inhibitors of these proteases (5). Recent studies have also demonstrated that TGF-β may play a pivotal role in the resistance of KFs to apoptosis (10, 12). However, there are no studies that we have noted on the molecular mechanisms of elevated TGF-β gene expression in keloids.**

As previously noted, keloids do not develop spontaneously, but rather as a result of injury, which can be anything from a surgical incision to an ear piercing to a tattoo (25). After acute injury, the wound healing program is initiated first with activation of the coagulation cascade. Cells in the immediate area of the wound are exposed to serum, which contains a number of cytokines and growth factors that propagate the wound-healing process. As an in vitro model, serum stimulation has been used for decades for studying cell growth control and cell cycle progression (19, 20, 27). In the standard serum stimulation protocol, fibroblasts are brought to a quiescent state (G0) by growth to confluence or by serum deprivation. The administration of serum to these cells stimulates them to reenter the cell cycle. In 1999, Iyer et al. (15) used cDNA microarrays to observe the temporal program of transcription of fibroblasts and noted that serum stimulation elicited a transcriptional response in fibroblasts similar to their response to injury by expressing numerous genes with known roles in the wound-healing process.

In this study, we have used serum stimulation as an in vitro model to mimic a component of the wound microenvironment to examine the transcriptional response to wounding in KFs compared with normal human fibroblasts (NFs). We hypothesized that KFs would have a differential response to serum stimulation vs. NFs by overexpressing TGF-β. Our data demonstrate that serum stimulation specifically and significantly upregulates TGF-β2 expression compared with other TGF-β isoforms in KFs but not in NFs. We further demonstrate that the p38 MAPK pathway is necessary for the differential TGF-β2 expression in KFs and that there is differential...
activation of components of the p38 MAPK pathway in KFs vs. NFs.

MATERIALS AND METHODS

Fibroblasts' primary culture and serum stimulation. KF primary cultures were derived from excised earlobe keloid specimens as previously described (21a). All patients (age range 14–21 yr) had received no previous treatment for the keloids before surgical excision. All specimens were obtained under approval from the Stanford University Institutional Review Board for Human Subjects Research (Protocol 76719). All experiments were repeated in three separate isolates of fibroblasts. NFs were from primary cultures of either tissues derived from repair of traumatically cleft earlobes (representing site-matched normal skin) or normal skin derived from breast biopsy specimens and foreskins. In all experiments, at least two of the controls were from carbole-derived cells. Only cells from the third to fifth passage were used for the experiments. All experiments were performed on each cellular isolate, and the data presented are the sum of those experiments. Serum stimulation was performed using the following protocol. Fibroblasts were grown to confluence and made quiescent in DMEM supplemented with 0.5% FBS for 48 h and then following protocol. Serum stimulation was performed using the protocol for TaqMan Human GAPDH Control Reagents (Applied Biosystems). Total RNA (1 μg) was reverse transcribed to cDNA using random hexamers per the manufacturer’s recommendation. Synthesized cDNA (1.5 μl) was served as substrate for PCR amplification of each interested gene. qPCR was performed in 384-well plates using specific primers and probes with the ABI PRISM 7900HT Sequence Detection System. Each sample was assayed in triplicate. Results were analyzed using Sequence Detection Systems v.1.6.3 software. The default settings of the program were used to define both the threshold value and baseline parameters for analysis of the raw data. The threshold cycle (CT) values of the samples were interpolated to an external reference curve constructed by plotting the relative amounts of a serial dilution of a known template vs. the corresponding CT values. The housekeeping gene GAPDH was used to normalize mRNA concentration.

Bioassay of conditioned medium for TGF-β activity. Conditioned medium from serum-stimulated fibroblasts was harvested at 1, 6, 12 and 24 h following stimulation. Cellular debris was removed by centrifugation (4°C, 1,000 g, 10 min). The protein concentration was determined using the BCA kit (Pierce, Rockford, IL). Activation of 1 ml conditioned medium was performed with 0.2 ml of 1 M HCl at room temperature for 10 min; the acidified sample was then neutralized by adding 0.2 ml of 1.2 M NaOH and 0.5 M HEPES. TGF-β concentration in the conditioned medium was assayed using the plasminogen activator inhibitor (PAI)-1/luciferase assay (1). Mink lung epithelial cells (MLECs) transfected with a truncated PAI-1 promoter luciferase assay (1). Mink lung epithelial cells (MLECs) transfected with a truncated PAI-1

Table 1. qPCR Primer and Probe Sequences

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Smad, family of signalling molecules triggered by TGF-β; COL1A1, collagen 1; F, 5’ primer; R, 3’ primer; P, labeled probe.
promoter fused to a firefly luciferase reporter gene were a generous gift from Dr. James Chang (Stanford University). MLECs were trypsinized, and the cell density was adjusted to $1 \times 10^5$ cells/ml with growth medium before adding $2 \times 10^5$ into each well of a six-well tissue culture plate (Falcon; Becton Dickinson, Franklin Lakes, NJ). Cells were incubated for 3–4 h at 37°C to allow attachment to the plastic. Following aspiration of the growth medium from the attached cells, 1 ml of the conditioned medium was added. All assays were performed in triplicate.

To generate standard curves for TGF-β activity and rule out the effect of various pathway inhibitors on MLECs, serial dilutions of recombinant human TGF-β1 (0 to 10 ng/ml) (R&D Systems, Minneapolis MN) in DMEM containing 10% FBS with or without each pathway inhibitor were added to the MLECs. Cells were then incubated with samples and TGF-β1 standards for 14–16 h at 37°C, 5% CO2. Following incubation, all wells were checked microscopically for cell viability before washing twice with 2 ml of PBS. Cells were then lysed using 500 µl/well of $1 \times$ lysis buffer (Luciferase Assay kit; Promega, Madison, WI) and incubated with agitation at room temperature for 20 min. The cell lysates (50 µl) were analyzed for luciferase activity according to the protocol provided by the manufacturer. The mean values of the triplicates were then converted into nanograms per milliliter using a standard curve obtained with human recombinant TGF-β1, normalized with the protein concentration of the conditioned medium.

Western blot analysis. Cell lysates were extracted at indicated time points following serum stimulation. Cells were rinsed twice with cold PBS and lysed in RIPA buffer (50 mM Tris pH 8.0, 1% Nonidet P-40, 150 mM NaCl, 0.5% deoxycholic acid, 0.1% SDS) with EDTA-free protease inhibitor cocktail (Boehringer Mannheim, Indianapolis, IN). Extracts were cleared by centrifugation (4°C, 14,000 g, 30 min), and the protein content was determined using the BCA kit (Pierce, Rockford, IL). Equal amounts of protein (100 µg/lane) in SDS loading buffer were electrophoresed on 12% SDS-PAGE. Proteins were then transferred onto Immobilon polyvinylidene difluoride membranes (Millipore, Bedford, MA) by electroblotting (Trans-Blot; Bio-Rad, Hercules, CA) in 25 mM Tris, 192 mM glycine, and 5% methanol at 90 V for 2 h. Membranes were probed with primary antibodies overnight at 4°C. After incubation with secondary antibody conjugated with horseradish peroxidase (HRP) [donkey anti-goat IgG-HRP (Santa Cruz Biotechnology, Santa Cruz, CA), goat anti-rabbit and goat anti-mouse IgG-HRP (Amersham Pharmacia Biotech, Piscataway, NJ)] for 1 h at room temperature, specific bands were revealed by incubation with the enhanced chemiluminescence solution Western blotting detection reagents (Amersham Pharmacia Biotech). Blots were exposed to high-performance chemiluminescence film (Amersham Pharmacia Biotech) for 0.5–10 min for detection. Filters were stripped by a 30-min incubation in 100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris·HCl (pH 6.8) at 50°C before reprobing with other antibodies. Antibodies to p38 MAPK were from Santa Cruz Biotechnology. Phospho-p90/RSK (Ser380), phospho-Erk1 (Ser383) antibody, phospho-activating transcription factor-2 (phospho-ATF-2) (Thr71) antibody, phospho-p38 MAPK (Thr180/ Tyr182) antibody, and phospho-Akt (Ser473) antibody were from Cell Signaling Technology, (Beverly, MA).

Statistical analysis. Data are expressed as means ± SE and are representative of experiments performed at least in triplicate in three separate cell isolations. Statistical evaluation of the data was performed using the unpaired Student’s t-test, considering $P$ values < 0.05 as significant.

RESULTS

Selective overexpression of TGF-β2 in KFs vs. NFs on serum stimulation. We compared the transcriptional response of TGF-β isoforms in KFs compared with NFs following serum stimulation. NFs and KFs were grown to confluence, made quiescent in DMEM supplemented with 0.5% FBS for 48 h, and stimulated with 10% FBS. Total RNA was harvested at indicated time points and subjected to qPCR analysis. The expression of each gene was normalized to the expression of housekeeping gene GAPDH and reported as expression relative to the baseline level, which is arbitrarily designated as one.

We found the mRNA level of TGF-β2 was rapidly upregulated and peaked between 1 and 6 h after serum stimulation in KFs, although it was not markedly changed in NFs (Fig. 1B). No differential transcriptional response was seen for TGF-β1 and -β3 (Fig. 1, A and C). We have also examined the mRNA and protein levels of other components of the TGF-β signaling pathway, including receptors and Smads, and have not found any significant differences between KFs and NFs (data not shown).

Serum stimulation leads to rapid transcriptional activation of TGF-β2 and increased functional activity. To determine whether the TGF-β2 induction in KFs possesses immediate early gene kinetics, we tested the expression of TGF-β2 with or without the presence of the protein synthesis inhibitor CHX.
TGF-β2 induction was not inhibited by the pretreatment of CHX in KFs, indicating that TGF-β2 induction was the direct response of KFs to serum stimulation without the requirement of de novo protein synthesis (Fig. 2A).

To correlate increased transcription of TGF-β2 with increased protein activity, we used MLECs containing a construct with the plasminogen activator inhibitor (PAI) promoter, a promoter responsive to TGF-β, driving transcription of luciferase to measure TGF-β activity levels in conditioned medium. We measured the amount of TGF-β activity normally found in serum and found it to be in the range of 5 ng/ml, consistent with previous reports of TGF-β activity in serum (data not shown), which we considered our baseline. We found a 2.5-fold increase from baseline in the active TGF-β level in conditioned medium from KFs 1 h after serum stimulation. There was no change of TGF-β concentration in conditioned medium from NFs following serum stimulation (Fig. 2B). This demonstrates that the increased transcription of TGF-β2 seen in KFs also leads to increased protein activity.

p38 MAPK is necessary for serum-induced TGF-β2 expression. We sought to define the signal transduction pathways involved in the serum induction of TGF-β2 expression of KFs. We blocked a number of signaling pathways that have been described (11, 16, 33) to be activated following serum stimulation by using small-molecule inhibitors of PI3-kinase (wortmannin), ERK1/2 (PD-98059), p38 MAPK (SB-203580), and JNK (SP-600125) to see if they could block the upregulation of TGF-β2. Among all of these inhibitors, only pretreatment of KFs with the p38 MAPK inhibitor SB-203580 was able to block the serum-induced upregulation of TGF-β2 (Fig. 3).

We used the MLEC PAI/luciferase system to measure the effects of signal transduction blockade on TGF-β activity levels in conditioned medium. To rule out any effect of these pathway inhibitors on induction of the PAI-1 promoter, we generated individual standard curves by plotting known recombinant human TGF-β1 concentrations with the presence of each pathway inhibitor vs. the corresponding light intensity (Fig. 4A). No significant differences were seen in the response to TGF-β in the presence of the different signal transduction inhibitors. We found that the increased TGF-β concentration observed in KFs following serum stimulation was only down-regulated by the p38 MAPK inhibitor SB-203580 (Fig. 4B). Although there is an increase in TGF-β activity at 12 h following serum stimulation in the presence of PD-98059, an ERK pathway inhibitor, the significance of this result is unclear. No significant effect was seen with inhibitors of other signal transduction pathways at any other time point.

Increased activation of p38 MAPK in KFs in response to serum stimulation. We hypothesized that if increased TGF-β2 transcription is mediated by the p38 MAPK pathway, we should be able to detect increased activation of that kinase. KFs and NFs were serum stimulated as described in MATERIALS AND METHODS, and total protein was harvested at a number of time points before immunoblotting. Total protein level of p38 MAPK did not change significantly before or after serum stimulation in both KFs and NFs. However, when we examined activation of p38 MAPK by assaying for levels of the phosphorylated protein, we found a significant difference. p38 MAPK was rapidly phosphorylated within 15 min, and phosphorylated protein could be detected out to 24 h after serum stimulation in KFs (Fig. 5). In NFs, the phosphorylation of p38 MAPK was of a much lower magnitude. We detect a faint band at 3 h with peak phosphorylation seen at 6 h and a return to
baseline by 24 h. This correlates with a minor increase in TGF-β2 transcription seen in NFs at 6 h.

Having detected increased activation of p38 MAPK, we would predict that transcription factors downstream of this signaling pathway would also show increased activation. We analyzed the phosphorylation of ATF-2 and Elk-1, two transcription factors that have been shown to be activated by p38 MAPK following serum stimulation (14, 33). Both ATF-2 and Elk-1 were phosphorylated within 15 min and were maintained at a high level in KFs to 6 h, while the phosphorylation was transient in NFs (Fig. 5). The increased phosphorylation of ATF-2 out to 6 h also corresponds with increased transcription of TGF-β2 out to 6 h.

The phosphorylation of the unrelated proteins pp90-Rsk and Akt are known targets for phosphorylation by ERK and PI3-kinases, two other signal transduction pathways activated following serum stimulation. Analysis of their phosphorylation levels showed no clear difference between KFs and NFs (Fig. 5), suggesting the enhanced activation is specific to the p38 MAPK pathway.

A corollary of our finding that blocking p38 MAPK prevents transcription of TGF-β2 is that this might lead to decreased collagen expression, one of the hallmarks of keloid disease. We examined expression of collagen 1 (COL1A1) at quiescence and 24 h after serum stimulation in the presence of multiple inhibitors of second messenger signaling pathways (Fig. 6).

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Fig. 4. Functional TGF-β activity in conditioned medium of KFs after treatment with second messenger pathway inhibitors. A: KFs were made quiescent and treated with known amounts of TGF-β1 in the presence of inhibitors of ERK, p38 MAPK, JNK, and PI3-kinase (50 μM PD-98059, 10 μM SB-203580, 25 μM SP-600125, and 100 nM wortmannin, respectively) to demonstrate that these molecules did not influence the response to TGF-β in the MLEC PAI/luciferase assay. No significant differences were seen with use of any inhibitor. B: KFs were made quiescent as before and were switched to media with inhibitors of ERK, p38 MAPK, JNK, and PI3-kinase (50 μM PD-98059, 10 μM SB-203580, 25 μM SP-600125, and 100 nM wortmannin, respectively) for 0.5 h before serum stimulation. Control samples without inhibitors had equal amounts of carrier (DMSO) added. Conditioned medium was harvested at indicated time points, and TGF-β concentration was analyzed by MLEC PAI/luciferase assay. Expression level at 0 h was designated as one. *P < 0.05.
We chose 24 h because that is when we have seen significant upregulation of collagen expression in keloid cells (data not shown). As predicted, treatment with p38 MAPK inhibitors inhibited upregulation of COL1A1. Interestingly, treatment with inhibitors of JNK inhibited COL1A1 to the same degree, whereas inhibitors of ERK led to increased COL1A1 expression. The finding that ERK inhibition led to increased COL1A1 expression is consistent with our earlier finding that ERK inhibition led to increased TGF-β activity in the conditioned medium.

DISCUSSION

Previous data have shown increased amounts of TGF-β protein in keloid tissue compared with normals (21, 28, 35). However, there are no molecular mechanisms described for why there is increased expression. Because keloids form only in response to injury, our hypothesis was that the increased expression of TGF-β was due, at least in part, to an exaggerated response to cellular stimuli that would promote proliferation and matrix deposition similar to what would happen during keloid formation. In this study, we have focused on the transcriptional regulation of TGF-β expression as a way to define a mechanism for the increased presence of the protein in keloid tissue. Because there are no animal models for keloid formation, we used the serum stimulation of primary cultured KFs compared with NFs as a method to expose the cells to a microenvironment similar to that which they would encounter during wound healing.

We found there is a differential, rapid transcriptional response of the TGF-β2 isoform, but not the β1 or β3 isoforms, in KFs compared with NFs. The induction of TGF-β2 occurred with immediate early kinetics and is associated with elevated TGF-β activity in conditioned medium of KFs after serum stimulation, suggesting that the increased transcription results in increased protein production and functional activity. Whereas there are minor increases in transcription of all three TGF-β isoforms in NFs, there is no concordant increase in measurable activity. We speculate that in addition to increased
transcription of TGF-β2, there may also be a difference in the production of functionally active protein.

We showed that the increased transcription of TGF-β2 was dependent on the p38 MAPK signaling pathway and proceeded to investigate the degree of p38 MAPK activation in KFs and NFs. Immunoblotting indicated that the total protein level of p38 MAPK did not change before or after serum stimulation in both KFs and NFs. In contrast, the phosphorylation of p38 MAPK was rapid and persistent in KFs after exposure to serum but very transient in NFs. Other downstream targets of p38 MAPK, Elk-1, and ATF-2 also demonstrated increased activation in KFs compared with NFs. Elk-1 is a transcription factor that has been shown to be activated by all three MAP kinases, whereas ATF-2 is phosphorylated by p38 MAPK and JNK (14, 33). In contrast, the phosphorylation of pp90/RSK and Akt, downstream targets of ERK and PI3-kinase, respectively, did not show a significant difference between KFs and NFs, suggesting that the differential activation of p38 MAPK is specific. Finally, we demonstrate that inhibition of p38 MAPK signaling pathways leads to decreased collagen I expression. The findings that JNK inhibition lead to a similar result and ERK inhibition leads to increased collagen I expression are intriguing, but it would be speculative for us to propose any mechanism for this as regulation of collagen I expression is complex.

The ERK, JNK, p38 MAPK, and PI3-kinase signaling pathways have been shown to play an instrumental role in transmission of signals from various environmental cues to the transcriptional machinery in the nucleus (11). Following exposure to extracellular stimuli, these signaling pathways induce a set of immediate early gene expression that represents the first major transcriptional response to these signals. Activation of this program leads to a variety of cellular responses, such as cell proliferation and differentiation, and derangements in this program leads to a variety of cellular responses, such as cell proliferation and differentiation, and derangements in this program can lead to pathology through oncogenic transformation (24). JNK and p38 MAPK are potently activated by proinflammatory cytokines and environmental stresses, such as UV irradiation and osmotic shock, and have been characterized as stress-activated protein kinases (16).

In accordance with our findings, there are studies demonstrating that the p38 MAPK cascade positively regulates TGF-β2 gene expression. A region of the TGF-β2 promoter contains two cis-regulatory elements thought to be necessary for transcription in response to cellular stresses: a CRE/ATF site positioned at −74 to −67 and an E-box motif located between −50 and −45 (17, 26, 30). Mutation of either site reduces the expression of TGF-β2 promoter/reporter gene constructs −60–80%. ATF-1 and -2 are capable of binding to the CRE/ATF motif, and upstream stimulatory factor (USF)-1 and -2 bind to the E-box element (17, 26, 30). The ATF and USF-1 transcription factors are both downstream targets of p38 MAPK (6, 13), and our current data demonstrate increased activation of ATF-2 in KFs compared with NFs.

Recently, many researchers also noticed the association of p38 MAPK in the pathogenesis of a variety of fibrotic diseases, including renal fibrosis, pulmonary fibrosis, cardiac fibrosis, pancreatic fibrosis, and liver fibrosis (2, 7, 8, 22, 23, 31, 34, 36). These studies indicate that blockade of p38 MAPK reduces inflammatory cytokines and extracellular matrix production and may be considered a potential therapeutic option in the treatment of fibrotic diseases (7, 22, 23, 31, 32).

Scleroderma patients have thickened skin that shares some similar pathologic features with keloids; there is a normal-appearing epidermis overlying thickened dermis although scleroderma is a diffuse process unrelated to wounding. One group reported TGF-β-induced type 1 collagen expression through p38 MAPK in sclerodema skin fibroblasts (29). However, they found the level and activity of p38 MAPK did not differ between scleroderma and healthy skin fibroblasts in response to TGF-β treatment. It is not clear whether the different findings are due to the different stimulation system employed in their study and our study or the inherent difference in gene regulation between keloid- and scleroderma-derived fibroblasts.

In conclusion, we have used in vitro serum stimulation of fibroblasts to simulate the in vivo response of fibroblasts to wounding. We demonstrate that KFs specifically upregulate TGF-β2 expression through activation of the p38 MAPK signal transduction pathway with resulting increased functional activity. The p38 MAPK pathway shows a keloid-specific increase in activation as measured by the amount of phosphorylated p38 MAPK and its downstream targets. Interestingly, previous data on activation of TGF-β2 in response to stress stimuli have identified p38 MAPK as the primary mediator of those signals. Our data provide a molecular mechanism for why keloids appear to elucidate more TGF-β and link the clinical disease of keloid formation with aberrations in signal transduction. Future studies seek to identify how p38 MAPK activation that is different in keloid fibroblasts and may provide specific targets for therapeutic intervention.

ACKNOWLEDGMENTS

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