11-11-2016

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Recommended Citation

Negmadjanov U, Holmuhamedov A, Emelyanova L, Xu H, Rizvi F, Ross GR, Tajik AJ, Shi Y, Holmuhamedov E, Jahangir A. TGF- β 1 increases resistance of NIH/3T3 fibroblasts toward apoptosis through activation of Smad2/3 and Erk1/2 pathways. J Patient Cent Res Rev. 2016;3:187-98. doi: [10.17294/2330-0698.1407](https://doi.org/10.17294/2330-0698.1407)

Published quarterly by Midwest-based health system Advocate Aurora Health and indexed in PubMed Central, the Journal of Patient-Centered Research and Reviews (JPCRR) is an open access, peer-reviewed medical journal focused on disseminating scholarly works devoted to improving patient-centered care practices, health outcomes, and the patient experience.

TGF- β 1 Increases Resistance of NIH/3T3 Fibroblasts Toward Apoptosis Through Activation of Smad2/3 and Erk1/2 Pathways

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Purpose	Excessive fibrosis has been suggested to result from persistence of fibroblasts in injured tissue due to impaired apoptosis, but signaling pathways are not fully defined.
Methods	Suppression of apoptotic cell death following transforming growth factor- β 1 (TGF- β 1) exposure was studied using the culture of NIH/3T3 mouse embryonic fibroblasts. Caspase-3 activity, propidium iodide staining and annexin V binding induced by Fas-ligand (FasL) in NIH/3T3 fibroblasts in the absence and presence of TGF- β 1 was determined, and relative contribution of signaling through Smad2/3 and noncanonical Erk1/2 and Akt pathways was dissected by assessing phosphorylation status of these kinases and caspase activity in the absence and presence of specific inhibitors (SB431542, PD0325901 and LY294002), respectively.
Results	TGF- β 1 treatment suppressed FasL-mediated fibroblasts apoptosis with a greater than threefold reduction of caspase-3 activity (from 894 ± 186 to 195 ± 56 nmol AFC/min/ 10^6 cells at 250 ng/mL of FasL) and reductions in cleaved caspase-8 and caspase-3 by 3.2-fold and 4.3-fold, respectively. The reduction in caspase activation was accompanied by a decrease in annexin V-positive cells by ~80%. TGF- β 1 treatment phosphorylated Smad2/3, Erk1/2 and Akt, which were reduced by their selective inhibitors. Inhibition of Smad2/3 and Erk1/2 alone partially reduced the protective effect of TGF- β 1 on caspase-3 activation, whereas inhibition of the Akt pathway had no significant effect. Concomitant inhibition of Smad2/3 and Erk1/2 completely reversed the protection by TGF- β 1.
Conclusions	TGF- β 1-mediated suppression of apoptosis in fibroblasts involves both Smad2/3 and Erk1/2 pathways, but not the Akt pathway. A combined approach inhibiting Smad2/3 and Erk1/2 pathways can completely reverse the protective effect of TGF- β 1 on apoptosis. These findings are proof of concept to help define strategies to reduce progression of fibrosis and resultant morbidities associated with conditions causing excessive fibrosis, including but not limited to keloid formation, transplant fibrosis and aging-associated fibrosis of the heart. (<i>J Patient Cent Res Rev.</i> 2016;3:187-198.)
Keywords	fibroblasts; TGF- β 1; stress kinases; Smad2/3; apoptosis; cell death; NIH/3T3; caspase; Erk1/2; Akt

Fibroblasts are the major cells involved in extracellular matrix remodeling and repair of injured tissue.¹⁻¹² Following extracellular matrix deposition, activated fibroblasts undergo programmed cell death or

dedifferentiate to quiescent phenotype, keeping the fibrotic process in check. Impairment of programmed cell death can result in persistence of these cells, which may contribute to progressive fibrosis and mechanical and electrical dysfunction in organs such as the heart, thereby predisposing a person to pathology that increases morbidity and mortality.¹²⁻¹⁴

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Apoptotic cell death and cell regulatory signaling cascades, as well as interactions between participating

proteins and cascades, are extremely complex.¹⁵ Programmed cell death is based on sequential activation of intracellular enzymes called caspases (stemming from cysteine-dependent, aspartate-directed proteases), a family of protease enzymes that play essential roles in initiation and progression of programmed cell death.¹⁶ Activation of crucial executional enzymes of this pathway, namely caspase-3, have been established as the key and executional protease commonly involved in all known mechanisms of extrinsic, intrinsic or mixed type of apoptotic cell death.¹⁷ It is important to understand mechanisms underlying excessive fibrosis and identify targets to prevent such complications to reduce morbidity and mortality associated with the growing epidemic of aging-associated diseases.^{2,4,12,14,18-23}

Transforming growth factor- β 1 (TGF- β 1), a profibrotic cytokine, has a variable effect on cell survival and proliferation depending on the cell type and clinical condition, with both proapoptotic and antiapoptotic effects previously described.²⁴ The effect of TGF- β 1 receptor activation on differentiation and maturation of fibroblasts is mediated through two intracellular signaling pathways: 1) canonical, Smad-dependent, and 2) noncanonical, Erk1/2-dependent. Smad-dependent signaling is specifically activated with TGF- β 1, a cytokine increased during inflammation, heart failure and other diseased states and which plays a crucial role in translation of cytoplasmic signals into the nucleus to induce activation of profibrotic transcription factors.²⁵ In addition, a noncanonical signaling pathway known as the Ras-Raf-MEK-Erk1/2 pathway, which involves a chain of intracellular proteins, transduces TGF- β 1 receptor activation on the surface of the cell to the DNA in the nucleus, thereby promoting cell proliferation, differentiation and migration.²⁶

Although both signaling pathways downstream of TGF- β 1 receptors are important in promoting fibrosis by proliferation and persistence of fibroblasts through inhibition of programmed cell death,^{27,28} their relative contribution to the antiapoptotic effect of TGF- β 1 is not well defined. It also is not clear whether inhibiting one of these downstream pathways alone is sufficient to reverse TGF- β 1's antiapoptotic effect. Therefore, we sought to determine the relative role of Smad-dependent and Smad-independent (i.e. Ras-Raf-MEK-Erk1/2 and Akt)

intracellular signaling pathways in TGF- β 1-mediated suppression of Fas ligand (FasL)-mediated apoptosis in an NIH/3T3 mouse embryo fibroblast cell line.

Identifying the relative contribution of canonical and noncanonical intracellular signaling in the mediation of the antiapoptotic effect of TGF- β 1 in fibroblasts may help design novel strategies to reduce progression of fibrosis and the resultant morbidity associated with conditions promoting this excessive tissue scarring.

METHODS

Materials

NIH/3T3 Mouse Embryonic Fibroblasts: NIH/3T3 cells (ATCC® CRL-1658™) were purchased from American Type Culture Collection (ATCC, Manassas, VA). Authentication of cells during propagation and long-term storage was verified using bright-field microscopy per ATCC recommendations (<https://www.atcc.org/~media/PDFs/Technical%20Bulletins/tb08.ashx>). Comparison of cellular morphology of intact, naive and TGF- β 1-treated cells was used as read-out of authenticity of NIH/3T3 cells susceptible to TGF- β 1-dependent maturation and differentiation followed by morphological changes from a naive triangular morphology with few processes in the absence of TGF- β 1 to mature differentiated cells that formed clearly identifiable “stripes” of large cells with multiple processes in the presence of TGF- β 1. These morphological changes were acquired by cells after at least 48 hours exposure to TGF- β 1 and retained by cell line at 60%–80% plating density (confluence). For the duration of this study, morphological checking of NIH/3T3 responsiveness to TGF- β 1 for each new passage and batch of cells was performed.

Chemicals: All chemicals were purchased from Sigma-Aldrich Corp. (St. Louis, MO) unless specified. Recombinant TGF- β 1 was obtained from PeproTech (Rocky Hill, NJ) and recombinant caspase-3 from Cell Signaling Technology Inc. (Danvers, MA). The caspase-3 substrate Ac(N-acetyl)-DEVD-7-amino-4-trifluoromethylcoumarin (Ac-DEVD-AFC) and AFC (for calibration) were obtained from Santa Cruz Biotechnology Inc. (Dallas, TX). FasL and antibodies against phospho-Smad2/3, Smad2/3, tubulin, phospho-Erk1/2, Erk1/2, phospho-Akt, Akt, procaspase-3, caspase-3, glyceraldehyde phosphate dehydrogenase (GAPDH) and secondary goat anti-

rabbit immunoglobulin G (IgG) were purchased from Cell Signaling Technology. Annexin V/Alexa Fluor® 488 dye mixture was obtained from Thermo Fisher Scientific Inc. (Waltham, MA). SB431542 was used as a specific inhibitor of Smad2; PD0325901, an MEK inhibitor, was used to inhibit Erk phosphorylation; and LY294002 was used as an inhibitor of the Akt pathway.

Cell Culture

NIH/3T3 fibroblasts were cultured in high-glucose DMEM media (ATCC) containing 10% bovine calf serum and 1% penicillin/streptomycin (both additives from Thermo Fisher Scientific) at 37°C in a humidified chamber supplemented with 5% carbon dioxide as previously described.¹¹ Cells of passage 3 were used after propagating from passage 0 obtained from vendor.

TGF-β1 Treatment

Cultured NIH/3T3 fibroblasts were treated with recombinant TGF-β1 as previously described.^{2,9,14,29-31} Briefly, cells were plated at the indicated density using complete cell culture media (10% fetal bovine serum) and incubated overnight at 95% air/5% carbon dioxide to allow attachment. Subsequently, cells were transferred into an incubation media with low serum (2.5%) for sensitization toward exogenous growth factors.^{11,32} Following 24 hours of sensitization, NIH/3T3 fibroblasts were further incubated for the indicated time in low-serum media supplemented with either 5 ng/mL of TGF-β1 (experimental group) or an appropriate volume of incubation media as a vehicle (control group). The workflow and setup used in these experiments are shown in Figure 1.

Measurement of Caspase-3

For caspase-3 activity assays, NIH/3T3 fibroblasts were grown in 96-well plates coated with rat tail collagen I (Sigma-Aldrich) at a density of 50,000 cells/cm². After 24 hours, media was replaced with 200 µl of low-serum incubation media (2.5% fetal bovine serum) and cells incubated for another 24 hours. After 24 hours, incubation media was replaced with 90 µl of low-serum media containing FasL (0.25–1 µg/ml) or vehicle. Following 6 hours of incubation, 90 µl of lysis buffer (HEPES 50 mM, CHAPS 10 mM, dithiothreitol 20 mM, pH 7.4) was added and the plate incubated at room temperature for 5 minutes. Cell lysates were then mixed with 100 µl of the caspase-3 reaction mixture containing HEPES 50 mM, sodium chloride 200 mM, ethylenediaminetetraacetic acid 2 mM, CHAPS 3 mM, dithiothreitol 20 mM and 10% sucrose and supplemented with highly specific caspase-3 substrate Ac-DEVD-AFC (50 µM). Caspase-3 cleaves the tetrapeptide between D and AFC, thus releasing the fluorogenic AFC, which was measured using Infinite® 200 PRO reader (Tecan Group Ltd., Männedorf, Switzerland) at 400 nm excitation and 505 nm emission. Commercial AFC was used for calibration of the fluorescence, and caspase-3 activity was expressed as nmol of coumarin/min/10⁶ cells.

Annexin V and Propidium Iodide Binding Assays

Fibroblasts, grown on collagen-coated glass surfaces of MatTek dishes, were exposed to TGF-β1 and treated with FasL. Following incubation with TGF-β1 and FasL, cells were washed with annexin binding buffer and then stained using a 1:10 mixture of annexin V and Alexa Fluor 488 dye (a fluorescent marker of

Figure 1. Diagram of experimental workflow. Cells were plated in complete incubation media (10% FBS) and incubated overnight (16–18 hours) to let fibroblasts attach. Cell sensitization was achieved by transferring the cells into low-serum media (2.5% FBS) for 24 hours. Following the sensitization period, cells were treated with TGF-β1 for 3 hours and exposed to FasL for another 6 hours. At that point cells were used for monitoring indicated parameters.

phosphatidylserine-enriched zones on the outer surface of the plasma membrane), 1 $\mu\text{g/ml}$ of Hoechst 33342 (a cell-permeant fluorescent nuclear dye for quantification of the cell number) and 10 $\mu\text{g/ml}$ propidium iodide (a plasma membrane-impermeable red nuclear fluorescing dye). Fluorescent images of NIH/3T3 fibroblasts were acquired in green (annexin V), blue (cell nuclei) and red (propidium iodide) channels using 40 $\times$ magnification, 0.5% power of excitation lines, and gain set at 850 for all three channels of the laser-scanning confocal microscope (FV1200, Olympus Corp., Waltham, MA). Confocal images of all three fluorophores were analyzed using ImageJ software. The total number of cells taken into analysis was determined from the total number of cell nuclei stained with Hoechst 33342 ($\lambda_{\text{ex/em}} = 350/461$ nm, blue fluorescence), the number of apoptotic cells was determined by the number of green cells labeled with annexin V/Alexa Fluor 488 ($\lambda_{\text{ex/em}} = 488/499$ nm), and the number of necrotic cells was derived from the number of propidium iodide-positive cells ($\lambda_{\text{ex/em}} = 535/617$ nm, red fluorescence).

Western Blotting

Activation of intracellular signaling molecules (Smad2/3, stress kinases Erk1/2, Akt), their phosphorylated forms and the active caspase-3 level in cells were assessed using western blot. Cultured fibroblasts were treated as described and solubilized using radioimmunoprecipitation assay (RIPA) buffer supplemented with a protease/phosphatase inhibitor cocktail per manufacturer's instructions (Sigma-Aldrich). Protein concentration of obtained cell lysates was determined using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific). Protein samples were mixed with NuPAGE® LDS Sample Buffer and NuPAGE Reducing Agent, heated at 70°C for 15 minutes, and the total of 15 μg of protein was loaded in each well of 4%–12% Bis-Tris gel (Thermo Fisher Scientific). The samples were separated at 110 V for ~2 hours using MOPS/SDS Running Buffer (Thermo Fisher Scientific). Separated proteins were then transferred to nitrocellulose membranes using the iBlot® system (Thermo Fisher Scientific).

Proteins of interest were detected using primary rabbit IgG antibodies to Smad2, Smad3, Erk1, Erk2, Akt, caspase-3, caspase-8, caspase-9, tubulin and GAPDH (Cell Signaling Technology), and goat anti-rabbit IgG

antibodies (Cell Signaling Technology) were used as secondary antibodies. The bands on the membrane were visualized using Clarity™ ECL Western Blotting Substrate (Bio-RAD, Hercules, CA), and images were taken using Amersham™ Imager 600 (GE Healthcare Life Sciences, Pittsburgh, PA). Western blot images were analyzed by ImageJ. Densities of bands of interest were normalized to the density of respective bands for GAPDH. Normalization to tubulin was used when molecular weight overlap was present between proteins of interest and GAPDH.

Statistical Analysis

Because no human or animal subjects were involved in this study, institutional review board approval was not required. Each experiment was performed in triplicate, with “n” representing the number of experiments. Data were expressed as means $\pm$ standard error of mean. Comparison between groups for each assay was performed using Student's t-test analysis, and $P < 0.05$ was considered to be statistically significant.

RESULTS

TGF- β 1 Treatment Desensitizes NIH/3T3 Fibroblasts to FasL-Induced Apoptotic Cell Death

To assess the effect of TGF- β 1 on survival against apoptosis induced by activators of extrinsic pathways, fibroblasts were incubated in the absence and presence of TGF- β 1 for 3 hours and treated with increasing doses of FasL (250–1,000 ng/ml). FasL induced activation of caspase-3 in a dose-dependent manner, with caspase-3 activity increasing from 232 ± 52 nmol AFC/min/ 10^6 cells at baseline to 895 ± 186 nmol AFC/min/ 10^6 cells at 250 ng/ml, 1618 ± 167 nmol AFC/min/ 10^6 cells at 500 ng/ml and $2,371 \pm 249$ nmol AFC/min/ 10^6 cells at 1,000 ng/ml of FasL ($n=3$, $P < 0.02$) (Figure 2A). TGF- β 1 pretreatment (5 ng/mL) reduced caspase-3 activation at all doses of FasL by more than threefold (from 895 ± 186 to 195 ± 56 nmol AFC/min/ 10^6 cells at 250 ng/ml of FasL, from 1618 ± 167 to 365 ± 65 nmol AFC/min/ 10^6 cells at 500 ng/ml of FasL and from $2,371 \pm 249$ to 696 ± 101 nmol AFC/min/ 10^6 cells at 1,000 ng/ml of FasL ($n=3$, $P < 0.03$) (Figure 2A). The dose of 250 ng/ml of FasL was used for further experiments (Figure 2B). Thus, FasL-induced caspase-3 activity was reduced by pretreatment of fibroblasts with TGF- β 1.

Figure 2. TGF- β 1 treatment desensitizes NIH/3T3 fibroblasts toward FasL-induced apoptotic cell death. **A:** Dose-dependent effect of FasL on activation of caspase-3 in NIH/3T3 cells in the absence (**open circles**) and presence (**closed circles**) of TGF- β 1 exposure (5 ng/ml for 3 hours; $n=3$, $P<0.02$ compared with non-TGF- β 1-treated cells). **B:** A quantitative representation of caspase-3 activation in NIH/3T3 fibroblasts treated with a single dose of FasL (250 ng/ml) in the absence (**open bar**) and presence (**closed bar**) of TGF- β 1 ($n=3$, $P<0.03$ compared with non-TGF- β 1-treated cells).

TGF- β 1-Mediated Reduction in FasL-Induced Caspase-3 Activation Is Associated With Reduced Apoptotic Cell Death

Treatment of NIH/3T3 fibroblasts with FasL (250 ng/ml) resulted in increased cell death as demonstrated by increased staining of nuclei with propidium iodide and increased annexin V binding (Figure 3A), indicative of apoptosis. At baseline in the absence of FasL, the number of propidium iodide- and annexin V-positive cells was negligible. FasL treatment resulted in $76\% \pm 17\%$ of cells staining with annexin V and $54\% \pm 22\%$ of cells staining with propidium iodide. Pretreatment of cells with TGF- β 1 reduced annexin V and propidium iodide staining (Figure 3A). On average, the percentage of annexin V-positive cells decreased from $76\% \pm 17\%$ in cells not exposed to TGF- β 1 to $15\% \pm 3\%$ in cells exposed to TGF- β 1 (5 ng/ml for 3 hours), demonstrating $\sim 80\%$ decrease of cell death by TGF- β 1 ($n=5$, $P<0.04$) (Figure 3B). Similarly, the percentage of propidium iodide-positive cells decreased from $54\% \pm 22\%$ in the absence of TGF- β 1 to $5\% \pm 4\%$ in the presence of TGF- β 1 ($n=5$, $P<0.05$) (Figure 3C). Thus, TGF- β 1 desensitized NIH/3T3 fibroblasts toward FasL-mediated cell death.

TGF- β 1 Prevents Activation of Caspase-3 and Cell Death by FasL Through Suppression of Caspase-8 Activation

To confirm that TGF- β 1 reduces FasL-mediated cell death through the extrinsic pathway of apoptosis that involves activation of caspase-8, the cleavage of caspase-3 and caspase-8 was assessed. Baseline expression levels of normalized cleaved caspase-3 and caspase-8 were negligible and not different in cells treated with or without TGF- β 1 (Figure 4). FasL treatment resulted in an approximately 23-fold increase of cleaved caspase-3 from baseline densitometric ratio (caspase-3 to tubulin) of 0.006 ± 0.001 to 0.120 ± 0.018 in fibroblasts (Figure 4A). Pretreatment of cells with TGF- β 1 before FasL exposure resulted in an approximately fourfold decrease in cleavage of caspase-3 in fibroblasts (densitometric ratio of caspase-3 to tubulin from 0.120 ± 0.018 in untreated cells to 0.030 ± 0.005 in TGF- β 1-treated cells; $n=3$, $P<0.01$). Similar results with an approximately threefold reduction of normalized cleaved caspase-8 levels were observed when FasL-treated cells were pretreated with TGF- β 1 (densitometric ratio of caspase-8 to GAPDH from 0.34 ± 0.05 in untreated cells to 0.11 ± 0.02 in treated cells; $n=3$, $P<0.01$) (Figure 4B). There was no difference

Figure 3. TGF-β1 treatment suppresses early signs of FasL-induced apoptotic cell death. **A:** Representative fluorescent confocal images of NIH/3T3 fibroblasts treated with FasL (250 μg/ml for 6 hours) in the absence (no TGF-β1) and presence (TGF-β1) of TGF-β1. Cells were stained with 1:10 dilution of annexin V/Alexa Fluor® 488 (green), 1 μg/ml of Hoechst 33342 (blue) and 50 μg/ml of propidium iodide (red). **B:** Bar graph of the percentage of annexin V/Alexa Fluor 488-positive cells in the population of NIH/3T3 fibroblasts treated with FasL in the absence (no TGF-β1) and presence (TGF-β1) of TGF-β1 (n=5, P<0.04). A total of 250 cells were quantified for each condition. **C:** Bar graph of the percentage of propidium iodide-positive cells in the population of NIH/3T3 cells treated with FasL in the absence (no TGF-β1) and presence (TGF-β1) of TGF-β1 (n=5, P<0.05). A total of 250 cells were quantified for each condition.

in procaspase-3 and procaspase-8 expression at baseline with FasL treatment or FasL combined with TGF-β1 treatment. Thus, pretreatment of NIH/3T3 fibroblasts with TGF-β1 (5 ng/ml for 3 hours) before induction of apoptotic cell death with FasL (250 ng/ml for 6 hours) decreased caspase-3 activation through suppression of caspase-8 cleavage.

TGF-β1 Treatment of NIH/3T3 Fibroblasts Activates Both Canonical and Noncanonical Intracellular Signaling Pathways

To further define the participation of canonical and noncanonical TGF-β1 receptor-activated pathways, we determined the phosphorylation of Smad2/3, Erk1/2 and Akt in the presence and absence of specific inhibitors in fibroblasts (Figure 5). TGF-β1 induced a 7.5-fold increase in phosphorylation of Smad2 (densitometric ratio of phospho-Smad2 to GAPDH from 0.04 ± 0.01 at baseline level to 0.30 ± 0.04 with TGF-β1 treatment), a twofold increase in Erk1/2 (densitometric ratio of phospho-Erk 1/2 to GAPDH from 0.03 ± 0.004 at baseline to 0.07 ± 0.01 with TGF-β1 treatment) and a 12-fold increase in Akt (densitometric ratio of phospho-Akt/Erk to tubulin from 0.02 ± 0.01 at the baseline level to 0.25 ± 0.03 with TGF-β1 treatment). Increased phosphorylation was inhibited by specific inhibitors of Smad2 (SB431542), Erk1/2

(PD0325901) and Akt (LY294002) pathways. Smad3 did not demonstrate substantial phosphorylation following TGF-β1 exposure (data not shown). TGF-β1-dependent activation of Smad2 was largely prevented by SB431542 (10 μM). SB431542 decreased the expression of phosphorylated Smad2 from 0.30 ± 0.04 to 0.08 ± 0.03 (densitometric ratio of phospho-Smad2 to GAPDH) in TGF-β1-treated fibroblasts (n=3, P<0.02) (Figure 5A). Similarly, PD0325901 (200 nM) decreased the expression of TGF-β1-mediated phosphorylated Erk1/2 from 0.07 ± 0.01 to 0.01 ± 0.01 (densitometric ratio of phospho-Erk 1/2 to GAPDH) (n=3, P<0.03), and LY294002 (5 μM) suppressed the ratio of phospho-Akt/tubulin from 0.25 ± 0.03 to 0.05 ± 0.02 (n=3, P<0.02) in TGF-β1-treated fibroblasts (Figure 5B, 5C).

TGF-β1-Dependent Suppression of FasL-Induced Apoptosis Is Reversed by Inhibiting Smad2/3 and Erk1/2 Pathways, but Not Akt

To elucidate the relative contributions of Smad-dependent and Smad-independent TGF-β1-mediated intracellular signaling pathways in protection against FasL-induced apoptosis, the effect of specific inhibitors on FasL-mediated activation of caspase-3 — the executioner of apoptotic cell death — was assessed. After 30 minutes of treatment with specific inhibitors, cells were

Figure 4. TGF-β1 treatment suppresses FasL-induced processing of procaspase-8 and procaspase-3 in NIH/3T3 cells. **A:** Western blot analysis of normalized caspase-3 (top) and procaspase-3 (bottom) levels in FasL-treated NIH/3T3 fibroblasts (250 ng/ml for 6 hours) in the presence and absence of TGF-β1 (5 ng/ml) ($n=3$, $P<0.01$). **B:** Western blot analysis of normalized caspase-8 (top) and procaspase-8 (bottom) levels in FasL-treated NIH/3T3 fibroblasts (250 ng/ml for 6 hours) in the presence and absence of TGF-β1 (5 ng/ml) ($n=3$, $P<0.01$) compared to FasL-treated cells without TGF-β1 treatment. CNT, carbon nanotube; NS, no significance.

exposed to TGF-β1 and then treated with FasL to initiate apoptotic cell death. In the absence of added inhibitors, TGF-β1 suppressed caspase-3 activity by fourfold ($n=4$, $P<0.02$) (Figure 6). This protective effect was reversed by pretreatment with SB431542, which restored the sensitivity of NIH/3T3 cell to FasL, resulting in caspase-3 activity increasing from 119 ± 35 nmol AFC/min/ 10^6 cells to 490 ± 88 nmol AFC/min/ 10^6 cells ($n=4$, $P<0.04$). Similarly, caspase-3 activity increased from 119 ± 35 nmol AFC/min/ 10^6 cells to 352 ± 63 nmol AFC/min/ 10^6 cells in the presence of PD0325901 ($n=4$, $P<0.05$). In contrast, LY294002 had no significant effect on TGF-β1-mediated protection (135 ± 26 nmol AFC/min/ 10^6 cells vs 119 ± 35 nmol AFC/min/ 10^6 cells; $n=4$, $P=0.51$). Since PD0325901 and SB431542 when used alone

partially restored the sensitivity of cells to FasL-induced apoptosis that had been suppressed by TGF-β1, SB431542 and PD0325901 were used concomitantly to assess the combinatory effect of inhibition of these two downstream pathways. Co-treatment with the two inhibitors in the presence of TGF-β1 completely restored the sensitivity of FasL-induced caspase-3 activity to 846 ± 123 nmol AFC/min/ 10^6 cells, similar to the levels in cells untreated by TGF-β1 ($n=4$, $P<0.01$) (Figure 6). Thus, TGF-β1 exposure reversibly suppressed FasL-induced activation of executioner caspase-3 through both canonical (Smad-dependent) and noncanonical (Smad-independent Erk1/2) intracellular signaling pathways. The protective effect of TGF-β1 on FasL-induced apoptosis was not affected by the Akt pathway.

Figure 5. TGF- β 1-induced activation of Smad2, Erk1/2 and Akt pathways was blocked by specific inhibitors of TGF- β 1 signaling pathway proteins in NIH/3T3 fibroblasts. **Top sections:** Western blots of Smad2 activation in cells treated for 3 hours with TGF- β 1 (5 ng/ml) only, or TGF- β 1 plus SB431542 (10 μ M) (A). Western blots of Erk1 and Erk2 activation in cells treated for 3 hours with TGF- β 1 only, or TGF- β 1 plus PD031542 (200 nM) in cells treated with FasL in the presence and absence of TGF- β 1 (B). Western blots of Akt activation in cells treated for 3 hours with TGF- β 1 only, or TGF- β 1 plus LY294004 (10 μ M) (C). **Bottom sections:** Bar graphs of the densities of normalized bands — open bars indicate untreated cells, closed bars indicate cells exposed to TGF- β 1, and hatched bars indicate cells exposed to TGF- β 1 in the presence of inhibitors ($n=3$ for each condition). * $P<0.02$ compared to cells treated without TGF- β 1; ** $P<0.03$ compared to cells treated with TGF- β 1.

DISCUSSION

The main finding of our study is that TGF- β 1 has a protective effect against FasL-mediated activation of apoptosis as shown by suppressed caspase-3 activity, reduced annexin V and propidium iodide staining and decreased cleaved caspase-3 and caspase-8 levels in NIH/3T3 fibroblasts. This protective effect was mediated through both Smad-dependent and Smad-independent Erk1/2 pathways that individually provided partial protection against FasL-mediated caspase activation. Specific inhibition of Smad2/3 or Erk1/2 phosphorylation alone only partially reversed TGF- β 1-mediated protection and concomitant inhibition of both the pathways completely reversed its protective effect. Although activated by TGF- β 1, the PI3K-Akt pathway was not involved in the protective effect of TGF- β 1 against FasL-mediated apoptotic cell death. These findings indicate that inhibition of both Smad2/3 and Erk1/2 pathways is required to completely block the protective effect of TGF- β 1 receptor activation, which could be clinically relevant for conditions of progressive fibrosis in which persistence of myofibroblasts is detrimental.

Figure 6. TGF- β 1-dependent suppression of FasL-induced apoptosis is reversed by inhibition of intracellular signaling pathways. FasL-induced activation of caspase-3 in naive and TGF- β 1-exposed NIH/3T3 cells in the absence (open bars) and presence (closed bars) of specific inhibitors of phosphorylation of Smad2/3 (SB431542, SB), Erk1/2 (PD0325901, PD) and Akt (LY294002, LY) and combination of Smad2/3 and Erk1/2 inhibition (SB+PD) ($n=4$, ^a $P<0.02$, ^b $P<0.04$, ^c $P<0.01$).

The role of receptor-mediated TGF- β 1 signaling in the regulation of cell growth, proliferation, differentiation and programmed cell death has been well documented in various cell types.^{1,2,11,13,18,22,23} There is also increasing evidence that signaling pathways downstream of TGF- β 1 receptors — both canonical Smad-dependent and noncanonical Smad-independent — are involved in mediating the effects of TGF- β 1, including its antiapoptotic effect.^{9,21,33–40} Our findings in NIH/3T3 fibroblasts indicate that inhibition of a single pathway only partially reverses the antiapoptotic effect of TGF- β 1, suggesting that both Smad-dependent and Erk1/2 pathways complement each other in suppression of apoptosis. The underlying basis for that is not completely understood and requires further investigation. Phosphorylation of Smad2/3 results in nuclear translocation of the Smad complex that regulates transcription of genes that ultimately translate into antiapoptotic effect.⁴¹ On the other hand, Erk kinases have a direct effect on preventing activation of caspase-8 and caspase-3.⁴² Thus, these two pathways complement each other to prevent program cell death mediated by FasL.

The role of Erk pathways in TGF- β 1-mediated suppression of apoptosis has been studied in macrophages, A-459 cells, NIH/3T3 and rat cardiac fibroblasts using serum deprivation, ceramide exposure and ischemia-reperfusion.^{38,40,43,44} Our findings extend these observations to FasL, a direct ligand for death receptor-induced apoptosis in NIH/3T3 fibroblasts. The role of Erk pathways in apoptosis has been previously demonstrated using NIH/3T3 cells overexpressing Bad, which resulted in inactivation of Erk pathways and reduced phosphorylated Erk compared to cells without Bad transfection that translated into enhanced sensitivity to ceramide- and serum deprivation-mediated apoptosis.⁴⁵ Furthermore, increased phosphorylation of Erk1/2 by TGF- β 1 correlated with the latter's protective effect against FasL.³⁸ Pharmacologic inhibition of Erk1/2 pathway by PD98059 also was shown to reduce apoptosis mediated by serum deprivation and ceramide production.³⁸ We confirmed this finding with a much more potent and highly sensitive inhibitor of MEK kinase, PD0325901.⁴⁶ Even at the highest concentration used in our study, only partial reversal (42%) of TGF- β 1-mediated suppression of apoptosis was observed, indicating that additional pathways are responsible for TGF- β 1-mediated protection.

Besides Erk1/2 phosphorylation, TGF- β 1 also caused phosphorylation of Akt and Smad2/3, which could be prevented by specific inhibitors. Smad2/3 phosphorylation induced by TGF- β 1 was suppressed by SB431542, a selective inhibitor of the TGF- β receptor kinase (TRK).⁴⁷ TRK inhibition results in decreased Smad2/3 phosphorylation, thus preventing downstream signaling and translocation of the Smad complex to the nucleus. Pretreatment with SB431542 partially reversed the protective effect of TGF- β 1 against FasL-mediated apoptosis (64%) as demonstrated by partial restoration of caspase-3 activity as compared to cells untreated by TGF- β 1. Concomitant inhibition of both Smad2/3 and Erk1/2 by SB431542 and PD0325901 pretreatment of NIH/3T3 fibroblasts prevented the protective effect of TGF- β 1 against FasL-mediated activation of executioner caspase-3 and apoptosis (Figure 6). Conversely, inhibition of the Akt pathway by LY294002, an effective inhibitor of Akt phosphorylation induced by TGF- β 1, had no impact on protective effect of TGF- β 1 against FasL-mediated caspase-3 activation, indicating this pathway is not critical for protection against activators of Fas-associated protein with death domain (FADD)-mediated cell death. Our data indicate that the main effectors of TGF- β 1-mediated suppression of apoptosis in NIH/3T3 cells are the Smad2/3 and Erk1/2 pathways, while the Akt pathway plays a minimal, if any, role in TGF- β 1-mediated suppression of FasL-induced apoptosis in NIH/3T3 fibroblasts, as illustrated in Figure 7.

Study Limitations

The current study was performed using an NIH/3T3 mouse embryonic fibroblast cell line as a proof-of-concept study to demonstrate that TGF- β 1-mediated desensitization of fibroblast apoptosis results from activation of intracellular signaling pathways that can be targeted to reduce persistence of fibroblasts that contribute to fibrosis progression. This needs to be confirmed in fibroblasts derived from patient samples to help define the utility of such an approach to reduce progression of fibrosis and resultant morbidities associated with conditions causing excessive fibrosis, including but not limited to keloid formation, transplant fibrosis and aging-associated fibrosis of the heart.

Figure 7. Schematic of TGF- β 1 receptor-mediated intracellular signaling pathways protecting against Fas-associated protein with death domain (FADD)-mediated caspase activation. Activation of FADD by binding of its ligand (FasL) through binding to death receptor activates caspase-8, which in turn activates executional caspase-3 and induces apoptotic cell death (blue lines). TGF- β 1 binding to its receptor activates downstream canonical and noncanonical signaling pathways. Phosphorylation of Smad2/3 results in formation of Smad complex (Smad2/3/4), which translocates to the nucleus and binds to Smad response elements to activate gene transcription, ultimately resulting in suppression of apoptosis. SB431542 inhibits TGF- β 1 receptor kinase and prevents Smad2/3 phosphorylation by TGF- β 1 (purple line). TGF- β 1 activates Ras protein with subsequent phosphorylation of Erk1/2. Activation of Erk1/2 inhibits caspase-3 and caspase-8 activation. PD0325901 selectively inhibits MEK kinase, thus preventing Erk1/2 phosphorylation (light blue line). TGF- β 1 signaling activates phosphatidylinositol-3-kinases (PI3K), resulting in phosphorylation of Akt; however, this pathway has no or minimal effect on protection against FasL-mediated apoptosis. LY294002 selectively inhibits Akt phosphorylation by inhibiting PI3K. Red lines indicate inhibition of caspases and broken lines indicate minimum involvement in TGF- β 1-mediated protection in NIH/3T3 fibroblasts.

CONCLUSIONS

Although the current study does not dissect the intimate details of TGF- β 1-mediated protection against FasL-induced apoptotic cell death, it provides strong evidence of involvement of both the Smad2/3 and Erk1/2 pathways, as well as noninvolvement of Akt, in this protective effect against extrinsic apoptosis in NIH/3T3 fibroblasts. Because fibroblasts

are the major cell types involved in extracellular matrix remodeling and their persistence can lead to excessive fibrosis that causes organ dysfunction with aging and aging-associated diseases, identifying targets that can antagonize the effect of cytokines and promote fibroblast removal is of potential clinical significance in reducing human morbidity from progressive fibrosis.

Patient-Friendly Recap

- Aging and its associated diseases can result in excessive scarring in different organs, thereby contributing to morbidity and death.
- The level of TGF- β 1 — a protein that contributes to scarring — increases in the setting of inflammation, heart failure and other disease states.
- Using mouse embryonic cell lines, the authors studied how three intracellular signaling pathways activated by TGF- β 1 might affect the cell death pathways responsible for scar formation.
- They found that the TGF- β 1 effect was mediated by two pathways, making them potential targets for the development of new drugs that may help prevent further scarring.

Acknowledgments

The authors express their gratitude to Milanka Petrovic and Kelsey Kraft of Aurora Research Institute (Milwaukee, WI) for their excellent technical support, and Brian Miller and Brian Schurrer of Aurora Cardiovascular Services for their help with figures. Ekhsan Holmuhamedov's current institution is the Institute of Theoretical and Experimental Biophysics, Russian Academy of Sciences (Pushchino, Moscow Region, Russia).

Conflicts of Interest

None.

Funding Sources

This work was supported by grants from the National Institutes of Health (R01-HL101240 and R01-HL089542 to Arshad Jahangir), and an intramural grant (to Ulugbek Negmadjanov) funded by the Aurora Health Care Foundation. The funders had no role in study design, data collection/analysis, decision to publish or preparation of the manuscript. The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIH.

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