

Collagen regulates transforming growth factor- β receptors of HL-1 cardiomyocytes through activation of stretch and integrin signaling

YEN-YU LU^{1,2}, YUNG-KUO LIN^{3,4}, YU-HSUN KAO^{5,6}, CHENG-CHIH CHUNG^{4,5},
YUNG-HSIN YEH⁷, SHIH-ANN CHEN^{8,9} and YI-JEN CHEN^{4,5}

¹Division of Cardiology, Department of Internal Medicine, Sijhih Cathay General Hospital, New Taipei City 221; ²School of Medicine, Fu-Jen Catholic University, New Taipei City 242; ³Division of Cardiology, Department of Internal Medicine, School of Medicine, College of Medicine, Taipei Medical University, Taipei 110; ⁴Division of Cardiovascular Medicine, Department of Internal Medicine, Wan Fang Hospital, Taipei Medical University, Taipei 116; ⁵Graduate Institute of Clinical Medicine, College of Medicine, Taipei Medical University, Taipei 110; ⁶Department of Medical Education and Research, Wan Fang Hospital, Taipei Medical University, Taipei 116; ⁷Cardiovascular Division, Chang Gung Memorial Hospital, Chang Gung University College of Medicine, Tao-Yuan 333; ⁸School of Medicine, National Yang-Ming University, Taipei 112; ⁹Division of Cardiology and Cardiovascular Research Center, Veterans General Hospital-Taipei, Taipei 116, Taiwan, R.O.C.

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Abstract. The extracellular matrix (ECM) and transforming growth factor- β (TGF)- β are important in cardiac fibrosis, however, the effects of the ECM on TGF- β signaling remain to be fully elucidated. The aims of the present study were to evaluate the role of collagen in TGF- β signaling and examine the underlying mechanisms. In the present study, western blot analysis was used to examine TGF- β signaling in HL-1 cells treated with and without (control) type I collagen (10 μ g/ml), which was co-administered with either an anti- β 1 integrin antibody (10 μ g/ml) or a stretch-activated channel inhibitor (gadolinium; 50 μ M). Cell proliferation and adhesion assays were used to investigate the roles of integrin, mechanical stretch and mitogen-activated protein kinases (MAPKs) on cell proliferation and adhesion. The type I collagen (10 μ g/ml)-treated HL-1 cells were incubated with or without anti- β 1 integrin antibody (10 μ g/ml), gadolinium (50 μ M) or inhibitors of p38 (SB203580; 3 μ M), extracellular signal-regulated kinase (ERK; PD98059; 50 μ M) and c-Jun N-terminal kinase (JNK; SP600125; 50 μ M). Compared

with the control cells, the collagen-treated HL-1 cells had lower expression levels of type I and type II TGF- β receptors (TGF β R1 and TGF β R2), with an increase in phosphorylated focal adhesion kinase (FAK), p38 and ERK1/2, and a decrease in JNK. Incubation with the anti- β 1 integrin antibody reversed the collagen-induced downregulation of the expression of TGF β R2 and phosphorylated FAK. Gadolinium down-regulated the expression levels of TGF β R1 and small mothers against decapentaplegic (Smad)2/3, and decreased the levels of phosphorylated p38, ERK1/2 and JNK. In addition, gadolinium reversed the collagen-induced activation of p38 and ERK1/2. In the presence of gadolinium and anti- β 1 integrin antibody, collagen regulated the expression levels of TGF β R1, TGF β R2 and Smad2/3, but did not alter the phosphorylation of p38, ERK1/2 or JNK. In addition, collagen increased cell proliferation and adhesion, and this collagen-induced cell proliferation was inhibited by the anti- β 1 integrin antibody and ERK inhibitor. Taken together, the data obtained suggested that collagen differentially regulated the expression levels of TGF β R1 and TGF β R2, and modulated the phosphorylation of MAPKs through integrin- or stretch-dependent and -independent signaling pathways.

Correspondence to: Dr Yi-Jen Chen or Dr Yung-Kuo Lin, Division of Cardiovascular Medicine, Department of Internal Medicine, Wan Fang Hospital, Taipei Medical University, 111 Hsin-Lung Road, Sec. 3, Taipei 116, Taiwan, R.O.C.
E-mail: yjchen@tmu.edu.tw
E-mail: yklin213@yahoo.com.tw

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Introduction

Cardiac fibrosis, as a consequence of remodeling processes, results from cardiac adaptations to the hemodynamic overload caused by heart failure and cardiac hypertrophy. Adaptive remodeling processes initiate structural alterations representing cell morphological changes and excessive deposition of extracellular matrix (ECM) (1-3). Substances in the ECM provide cardiomyocytes with structural, chemical and mechanical substrates, which are essential for normal development and

responses to pathological signals. Cardiomyocytes can sense stimuli from the ECM, which results in structural or functional remodeling (4). Collagen, particularly type I, is a major component of the ECM, which forms networks in interstitial spaces among cells, and provides structural integrity and mechanical stretching of the heart (5). Therefore, type I collagen may be critical in cardiac fibrosis.

Transforming growth factor (TGF)- β is a key mediator in the pathogenesis of hypertrophic and dilative ventricular remodeling by stimulating cardiomyocyte growth and inducing interstitial fibrosis (6,7). In the pressure-overloaded human heart, the upregulation of TGF- β is correlated with the degree of fibrosis (8). The overexpression of TGF- β in transgenic mice has been found to result in atrial hypertrophy, which is characterized by interstitial fibrosis and hypertrophic growth of cardiomyocytes (9). Active TGF- β binds to the constitutively active type II receptor (TGF β RII) at the cell surface, and subsequently transphosphorylates the cytoplasmic domain of the type I receptor (TGF β RI) (10). As TGF β RI mediates the majority of the cellular responses to TGF- β_1 and has a predominant role in intracellular signal transduction (11), the activation of TGF- β receptors propagates downstream intracellular signals through Smad proteins, which are essential components in the signaling pathway of TGF- β (10,12-14). Although TGF- β signaling is important in genes associated with the ECM, whether the ECM is involved in regulating TGF- β signaling remains to be elucidated.

In vivo, cardiomyocytes are intricately connected to the ECM, and stretch is transduced via the intercellular and ECM connections via proteins located on the cell surface and subcellular adhesion complexes, and via stretch-sensing proteins. Stretching and the expression of integrin are increased during cardiac fibrosis (15-17). Integrin represents a primary link between ECM ligands and directly initiates intracellular signaling cascades (18-21). Integrin can interact with TGF- β to enhance cardiac fibrosis (21), suggesting that collagen may be involved in TGF- β signaling, which regulates the homeostasis of cardiac fibrosis. Integrin can activate focal adhesion kinase (FAK), which results in cardiac structural and electrical changes (22). In addition, mechanical stretch is important in tissue morphogenesis and remodeling, and evokes various hypertrophic responses from cell adhesion receptors and the expression of TGF- β_1 , and activates mitogen-activated protein kinases (MAPKs) in cardiomyocytes (23-25). Accordingly, integrin and mechanosensitive stretch channels may potentially regulate TGF- β signaling. MAPKs have pathophysiological effects in cardiac remodeling (26). Our previous studies demonstrated that collagen can modulate calcium dynamics and electrical activities of cardiomyocytes, with activation of the renin-angiotensin and MAPK systems (27,28). Therefore, the aims of the present study were to evaluate the effects of collagen on TGF- β signaling and investigate the underlying mechanisms. The results of the present study demonstrated that collagen differentially modulated the expression levels of TGF β RI and TGF β RII and the activity of MAPKs through interactions of integrin with stretch signaling.

Materials and methods

Cell culture. The protocol was approved by the Department of Medical Research of Cathay General Hospital, Taipei, Taiwan (no. CGH-MR-10014). HL-1 cells derived from

mouse atrial cardiac muscle cells (29) were provided by Dr Claycomb, Louisiana State University Health Sciences Center (New Orleans, USA). The cells for various experiments were cultured in a humidified atmosphere of 5% CO₂ at 37°C in Claycomb medium (JRH Biosciences, Lenexa, KS, USA). As described previously, the HL-1 cells were cultured with or without (control) type I rat tail tendon collagen (10 μ g/ml; Sigma-Aldrich, St. Louis, MO, USA) for 24 h following cell seeding (27). To investigate the roles of integrin and stretch, the HL-1 cells were pretreated with either monoclonal hamster anti-rat β 1 integrin antibody (β 1 integrin; 10 μ g/ml; clone Ha2/5; #555002; BD Pharmingen, San Diego, CA, USA), to inhibit the *in vitro* adhesion of CD29-expressing cells to type I collagen, or the stretch-activated ion channel inhibitor, gadolinium (50 μ M; Sigma-Aldrich) for 30 min prior to collagen incubation (30,31). The cells were plated at a density of 5x10⁵ cells/well in 6-well culture plates.

Western blot analysis. The HL-1 cells were homogenized and lysed in RIPA buffer containing 50 mM Tris (pH 7.4), 150 mM NaCl, 1% NP40, 0.5% sodium deoxycholate, 0.1% sodium dodecylsulfate (SDS) and protease inhibitor cocktails (Sigma-Aldrich). The protein concentrations were determined using Bio-Rad protein assay reagent (Bio-Rad Laboratories, Inc., Hercules, CA, USA). Protein samples (40 μ g) were separated by 10% SDS-polyacrylamide gel electrophoresis under reducing conditions and electrophoretically transferred onto equilibrated polyvinylidene difluoride membranes (Amersham Biosciences, Uppsala, Sweden). The blots were probed with the following primary antibodies overnight at 4°C: Rabbit anti-FAK (cat. no. 3285; 1:1,000), rabbit anti-phosphorylated FAK (cat. no. 3283; 1:1,000), rabbit anti-extracellular signal-regulated kinase 1/2 (ERK1/2; cat. no. 9102; 1:1,000), rabbit anti-phosphorylated-ERK1/2 (cat. no. 4370; 1:1,000) rabbit anti-c-Jun N-terminal kinase (JNK; cat. no. 9258; 1:1,000), mouse anti-phosphorylated-JNK (cat. no. 9255; 1:1,000), rabbit anti-p38 (cat. no. 9212; 1:1,000), rabbit anti-phosphorylated-p38 (cat. no. 9211; 1:1,000), rabbit anti-Smad2/3 (cat. no. 8685; 1:1,000), rabbit anti-phosphorylated-Smad2/3 (cat. no. 8828; 1:1,000), rabbit anti-TGF β RII (cat. no. 11888; 1:500) (all Cell Signaling Technology, Inc., Danvers, MA, USA); rabbit anti-TGF β RI (cat. no. SAB1300113, Sigma-Aldrich; 1:500); and horseradish peroxidase-conjugated goat anti-rabbit (cat. no. AP132P; 1:20,000) or goat anti-mouse immunoglobulin G (cat. no. AP124P; 1:8,000) secondary antibodies (EMD Millipore, Billerica, MA, USA) for 1 h at room temperature. The bound antibodies were detected with an enhanced chemiluminescence detection system (EMD Millipore) and analyzed with AlphaEaseFC version 4.0 software (Alpha Innotech Corporation, San Leandro, CA, USA). The targeted bands were normalized to mouse anti-cardiac α -sarcomeric actin (cat. no. A2172; Sigma-Aldrich; 1:2,000) to confirm equal protein loading.

Cell proliferation assay. Cell proliferation was analyzed using a BrdU incorporation assay (BrdU Cell Proliferation Assay kit, Cell Signaling Technologies, Inc.). The HL-1 cells were seeded at 1x10⁴ cells/well in 96-well plates and left to attach overnight. The cells were then incubated with or without

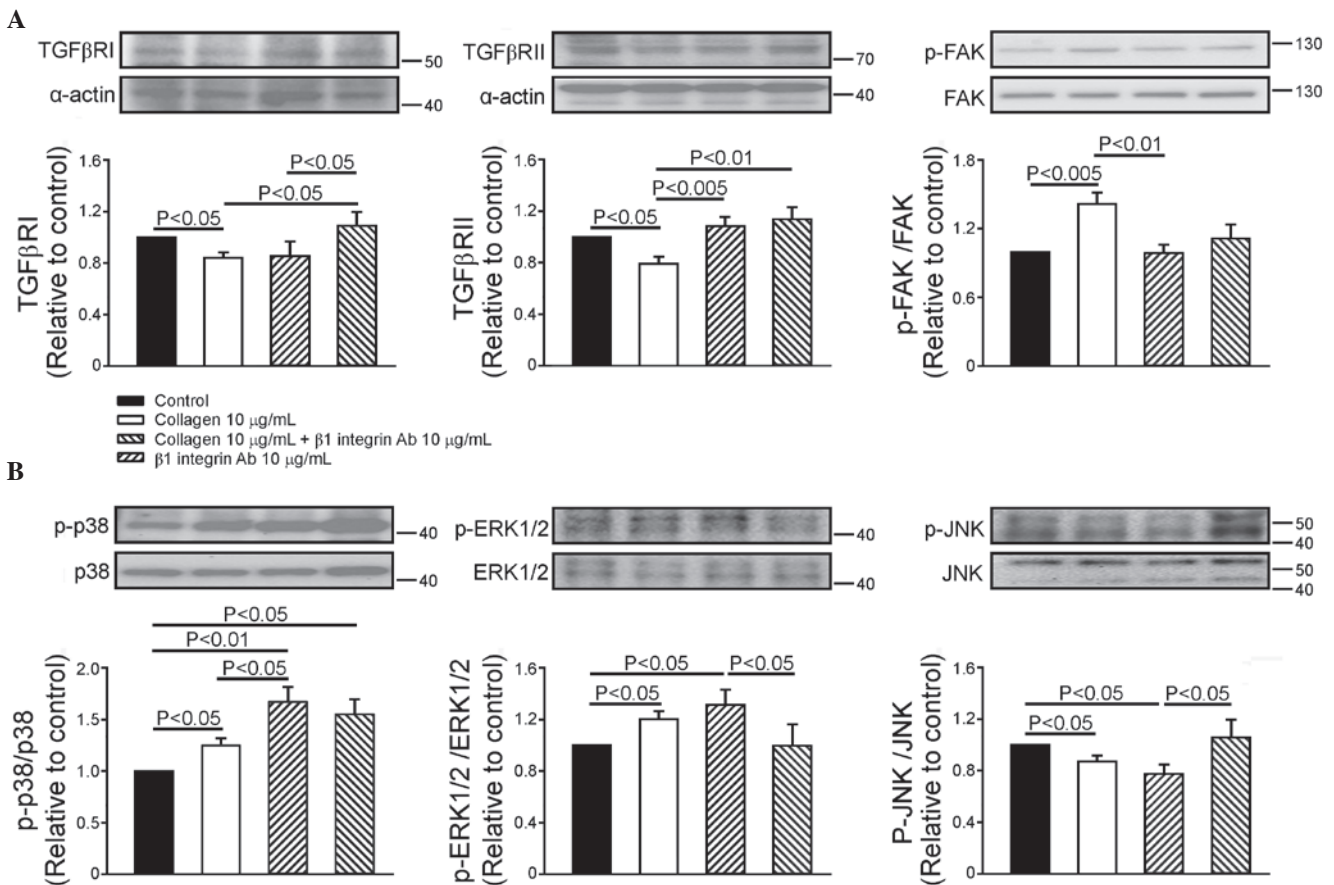


Figure 1. Effects of collagen and $\beta 1$ integrin Ab on the expression levels of TGF β RI and TGF β RII, and the downstream signaling in HL-1 cells. (A) Representative images and quantified data (n=7) of the expression levels of TGF β RI and TGF β RII, and the phosphorylation ratio of FAK in the control, collagen (10 μ g/ml)-treated, collagen (10 μ g/ml) with $\beta 1$ integrin Ab (10 μ g/ml)-treated, and $\beta 1$ integrin Ab (10 μ g/ml)-treated groups. (B) Representative image and quantified data (n=6) of the phosphorylation ratio of p38, ERK1/2 and JNK in the control, collagen (10 μ g/ml)-treated, collagen (10 μ g/ml) with $\beta 1$ integrin Ab (10 μ g/ml)-treated and $\beta 1$ integrin Ab (10 μ g/ml)-treated groups. Data are presented as the mean \pm standard error of the mean. TGF β R, transforming growth factor β receptor; FAK, focal adhesion kinase; ERK1/2, extracellular-regulated kinase 1/2; JNK, Jun N-terminal kinase; Ab, antibody; p-, phosphorylated.

type I rat tail tendon collagen (10 μ g/ml) for 24 h. The HL-1 cells were pretreated with $\beta 1$ integrin antibody (10 μ g/ml), gadolinium (50 μ M), p38 MAPK inhibitor (SB203580; 3 μ M; Merck Millipore, Darmstadt, Germany), ERK inhibitor (PD98059; 50 μ M; Sigma-Aldrich) or JNK inhibitor (SP600125; 50 μ M; Calbiochem, San Diego, CA, USA) for 30 min prior to collagen incubation. BrdU solution was added 4 h prior to the end of cell treatment. Following fixing of cells and denaturing of DNA, BrdU detection antibody and horseradish peroxidase-conjugated secondary antibody were added. Finally, the HRP substrate, 3,3',5,5'-tetramethylbenzidine, was added to develop color, and BrdU incorporation was quantified by reading the absorbance at 540 nm.

Cell adhesion assay. The HL-1 cells were trypsinized and seeded at a density of 2×10^4 cells/well in a 96-well plate following treatment with type I collagen (10 μ g/ml) and $\beta 1$ integrin antibody (10 μ g/ml). Subsequent to attachment for 2.5 h in a 37°C incubator, the unattached cells were removed by washing with phosphate-buffered saline. The cells were then stained with 0.1% crystal violet in 2% ethanol in 0.1 M borate (pH 9) for 15 min. Cell adhesion levels were evaluated by reading the absorbance of crystal violet at 540 nm following the addition of 100 ml pure methanol to solubilize the dye.

Statistical analysis. Continuous variables are expressed as the mean \pm standard error of the mean. The different groups of HL-1 cells were compared using a paired *t*-test or repeated one-way analysis of variance with a Tukey's post-hoc test. The effects of the $\beta 1$ integrin antibody or gadolinium on collagen were evaluated using an unpaired *t*-test. All statistical tests were performed using SigmaStat 3.5 (Systat Software, Inc., San Jose, CA, USA). $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Effects of collagen and $\beta 1$ integrin in modulating the expression of TGF- β receptors and FAK. Compared with the control, the collagen (10 μ g/ml)-treated HL-1 cells exhibited significantly decreased expression levels of TGF β RI (16 \pm 5%) and TGF β RII (21 \pm 5%) and upregulated FAK phosphorylation, (42 \pm 9%). The $\beta 1$ integrin antibody (10 μ g/ml) did not alter the expression levels of TGF β RI, TGF β RII or the phosphorylation of FAK in the HL-1 cells. In the presence of the $\beta 1$ integrin antibody, collagen downregulated the expression of TGF β RI, however, no effects on the expression of TGF β RII or the phosphorylation of FAK were observed (Fig. 1A). Therefore, the $\beta 1$ integrin antibody attenuated the collagen-associated downregulation in the expression of TGF β RII and phosphorylation

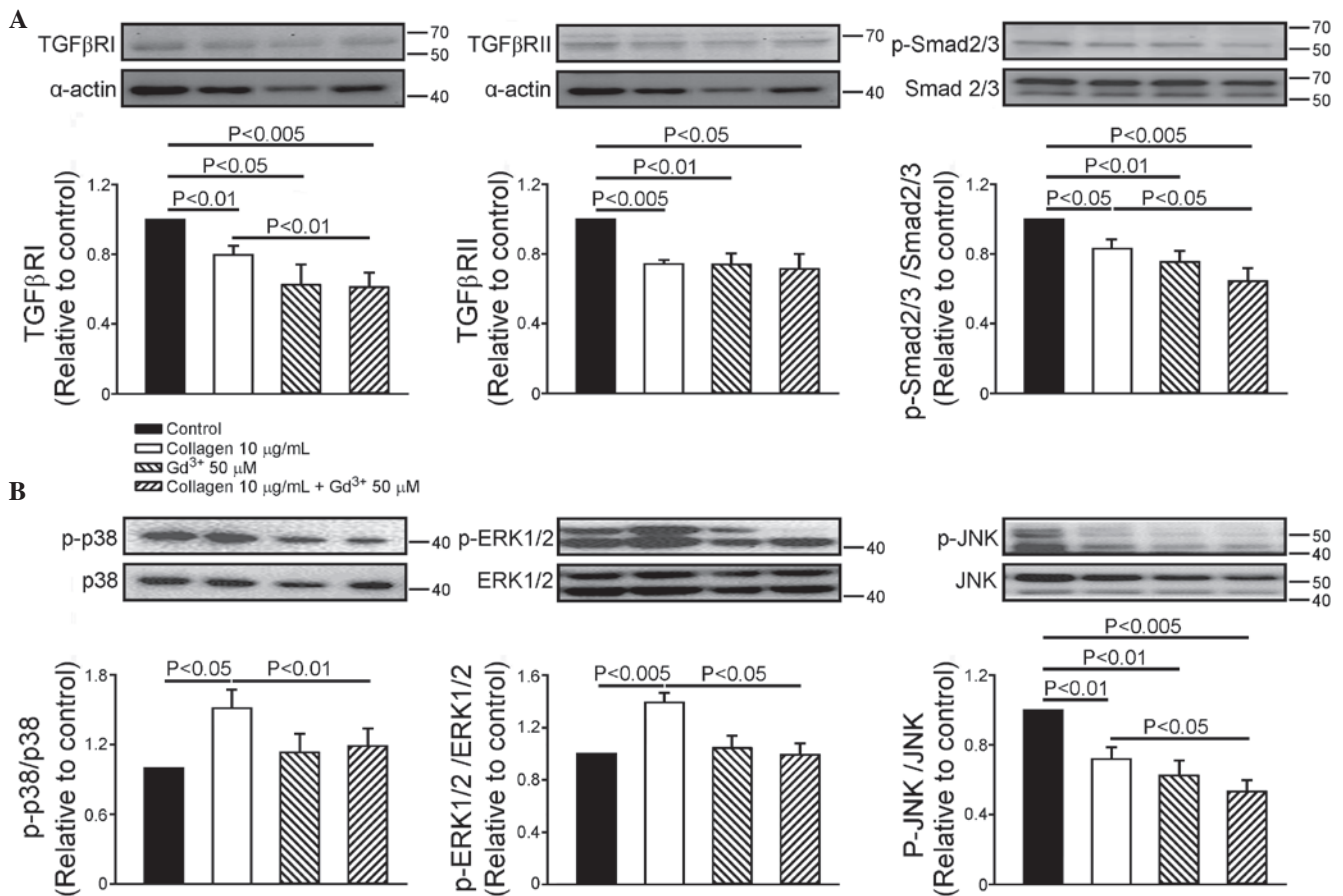


Figure 2. Effects of Gd³⁺ on TGF β RI, TGF β RII and Smad2/3, and the phosphorylation of mitogen-activated protein kinases. (A) Representative images and quantified data of TGF β RI, TGF β RII and Smad2/3 (n=7) and (B) phosphorylation ratios of p38, ERK1/2 and JNK (n=6) in the control, collagen (10 μ g/ml)-treated, Gd³⁺ (50 μ M)-treated and collagen (10 μ g/ml) with Gd³⁺ (50 μ M)-treated groups. Data are presented as the mean \pm standard error of the mean. Gd³⁺, gadolinium; TGF β R, transforming growth factor β receptor; ERK1/2, extracellular-regulated kinase 1/2; JNK, Jun N-terminal kinase; Smad, small mothers against decapentaplegic; Ab, antibody; p-, phosphorylated.

of FAK, but did not alter the collagen-associated downregulation of TGF β RI.

Effects of collagen and β 1 integrin in the modulation of MAPK phosphorylation. As shown in Fig. 1B, the collagen (10 μ g/ml)-treated HL-1 cells exhibited increased phosphorylation of p38 (25 \pm 7%) and ERK1/2 (20 \pm 6%), but decreased the phosphorylation of JNK (13 \pm 5%). The β 1 integrin antibody increased the phosphorylation of p38 by 55 \pm 15% in the HL-1 cells, but did not affect the phosphorylation of ERK1/2 or JNK. Therefore, β 1 integrin was expected to increase the level of phosphorylated p38. In the presence of the β 1 integrin antibody, collagen did not alter the phosphorylation of p38, however, the phosphorylation of ERK1/2 was increased and that of JNK was decreased. Accordingly, the collagen-induced changes in the levels of phosphorylated ERK1/2 and JNK were not altered by the presence of the β 1 integrin antibody, which suggested that integrin may not completely modulate the collagen effects of collagen on MAPKs.

Effect of mechanical stretch in collagen-mediated expression of the TGF- β receptor and Smad2/3. Compared with the control, gadolinium (50 μ M) decreased the expression of TGF β RI by 38 \pm 12% and TGF β RII by 26 \pm 6%. In the presence

of gadolinium, collagen did not alter the expression levels of TGF β RI or TGF β RII. However, gadolinium decreased the collagen-induced downregulation of TGF β RI by 25 \pm 7%. Collagen decreased the phosphorylation of Smad2/3 by 17 \pm 5%, compared with the control. Gadolinium (50 μ M) decreased the phosphorylation of Smad2/3 in the HL-1 cells by 24 \pm 6%, and decreased the phosphorylation of Smad2/3 by 23 \pm 6% in the presence of collagen (Fig. 2A).

Effect of mechanical stretch on the collagen-mediated phosphorylation of MAPKs. Compared with the control cells, the gadolinium (50 μ M)-treated HL-1 cells exhibited decreased JNK phosphorylation by 37 \pm 6%, however, the phosphorylation of p38 or ERK1/2 were not affected. In the presence of gadolinium, collagen did not alter the phosphorylation of p38, ERK or JNK. However, gadolinium further decreased the collagen-induced downregulated phosphorylation of JNK by 25 \pm 8%, and reversed the collagen-induced upregulated phosphorylation of p38 and ERK1/2, compared with the collagen-treated HL-1 cells (Fig. 2B).

Effects of β 1 integrin and mechanical stretch on the collagen-mediated expression of TGF- β receptor and Smad2/3. As shown in Fig. 3A, the β 1 integrin antibody (10 μ g/ml) and gadolinium (50 μ M) decreased the expres-

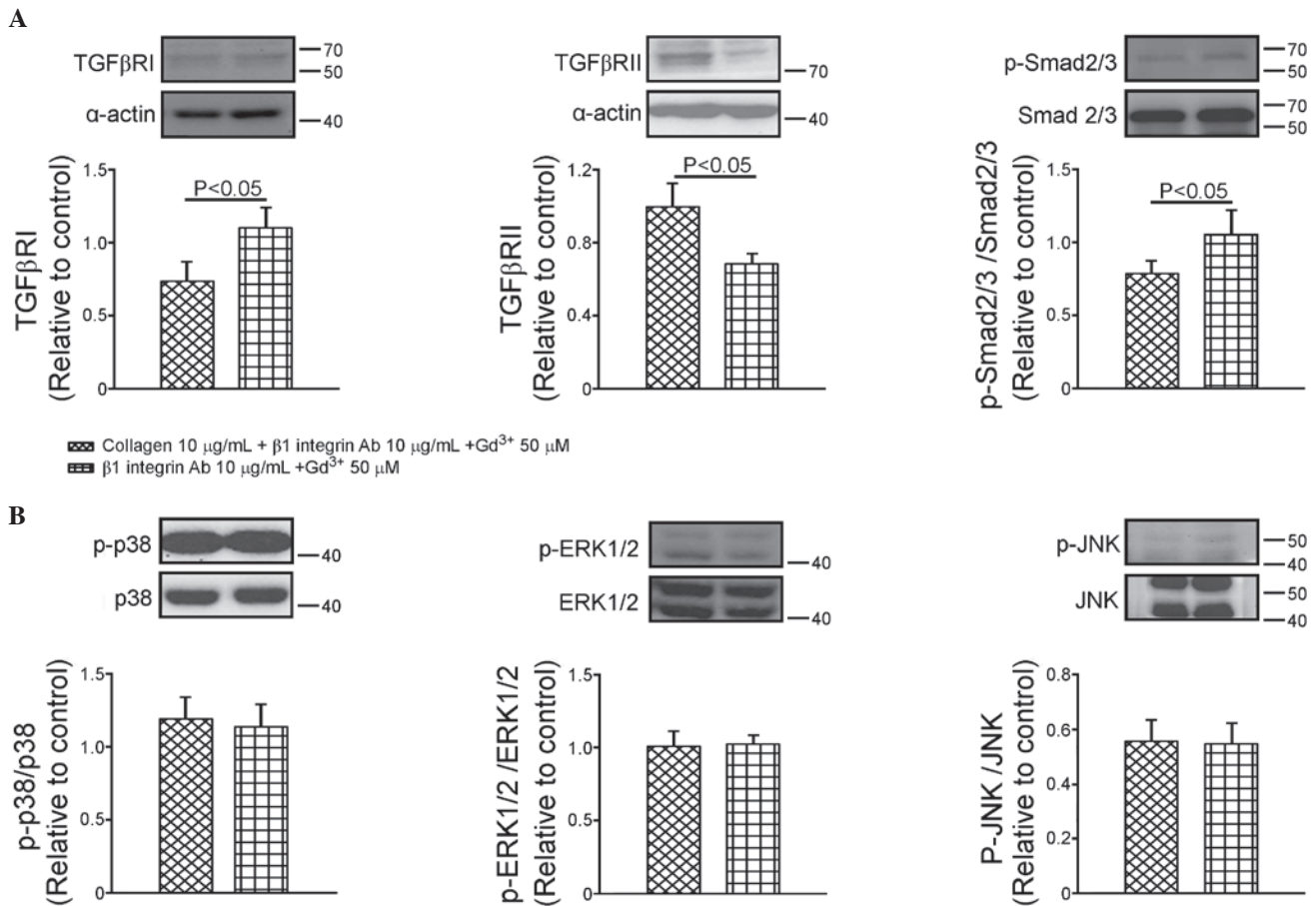


Figure 3. Effects of $\beta 1$ integrin Ab and Gd^{3+} on the expression of TGF β RI, TGF β RII and Smad2/3 and the phosphorylation of mitogen-activated protein kinases. (A) Representative images and quantified data (n=7) of TGF β RI, TGF β RII and Smad2/3 in the groups treated with collagen, $\beta 1$ integrin Ab (10 μ g/ml) and Gd^{3+} (50 μ M), and with $\beta 1$ integrin Ab (10 μ g/ml) and Gd^{3+} (50 μ M). (B) Representative images and quantified data (n=6) of the phosphorylation ratios of p38, ERK1/2 and JNK in the groups treated with the collagen, $\beta 1$ integrin Ab (10 μ g/ml) and Gd^{3+} (50 μ M), and with $\beta 1$ integrin Ab (10 μ g/ml) and Gd^{3+} (50 μ M). Data are presented as the mean \pm standard error of the mean. Gd^{3+} , gadolinium; TGF β R, transforming growth factor β receptor; FAK, focal adhesion kinase; ERK1/2, extracellular-regulated kinase 1/2; JNK, Jun N-terminal kinase; Ab, antibody; p-, phosphorylated.

sion of TGF β RII by $32 \pm 6\%$ ($P < 0.05$, vs. control), however, no change in the expression of TGF β RI was observed in the HL-1 cells. In the presence of the $\beta 1$ integrin antibody and gadolinium, collagen downregulated TGF β RI by $66 \pm 23\%$ and upregulated TGF β RII by $26 \pm 8\%$. Compared with the gadolinium-treated cells (Fig. 2A), the HL-1 cells treated with the $\beta 1$ integrin antibody and gadolinium (Fig. 3A) increased the expression of TGF β RI by $149 \pm 77\%$ ($P < 0.05$), however, the expression of TGF β RII was not affected (data not shown). The phosphorylation of Smad2/3 in the cells treated with the $\beta 1$ integrin antibody and gadolinium did not differ from that of the control. The effects of the $\beta 1$ integrin antibody and gadolinium on the phosphorylation of Smad2/3 were similar to those on the expression of TGF β RI.

Effects of $\beta 1$ integrin and mechanical stretch in the collagen-mediated expression of MAPKs. Compared with the control HL-1 cells, $\beta 1$ integrin antibody (10 μ g/ml) and gadolinium (50 μ M) decreased the phosphorylation of JNK by $45 \pm 8\%$ ($P < 0.005$), but did not alter the phosphorylation of p38 or ERK1/2 (Fig. 3B). In the presence of $\beta 1$ integrin antibody and gadolinium, no changes in the phosphorylation of p38, ERK1/2 or JNK were observed, compared with the

gadolinium-treated cells and the cells concomitantly treated with collagen.

Effects of $\beta 1$ integrin and mechanical stretch on the proliferation and adhesion of collagen-treated HL-1 cells. To investigate the role of integrin and mechanical stretch on cell proliferation, the collagen (10 μ g/ml)-treated HL-1 cells were incubated with or without $\beta 1$ integrin antibody (10 μ g/ml) or gadolinium (50 μ M). As shown in Fig. 4A, collagen increased cell proliferation, and this collagen-induced cell proliferation was inhibited by $\beta 1$ integrin antibody, but not by gadolinium. In addition, collagen increased cell adhesion by $16 \pm 7\%$, which was also inhibited by $\beta 1$ integrin antibody (Fig. 4B). These results suggested that collagen promoted cardiomyocyte proliferation and adhesion through $\beta 1$ integrin signaling.

Effect of MAPK inhibition on the proliferation of collagen-treated HL-1 cells. Compared with the control HL-1 cells, SB203580 (3 μ M) increased cell proliferation by $33 \pm 11\%$, whereas PD98059 (50 μ M) and SP600125 (50 μ M) did not alter cell proliferation (Fig. 5). However, in the presence of collagen, PD98059 and SP600125, but not SB203580, inhibited the collagen-induced increase in cell proliferation.

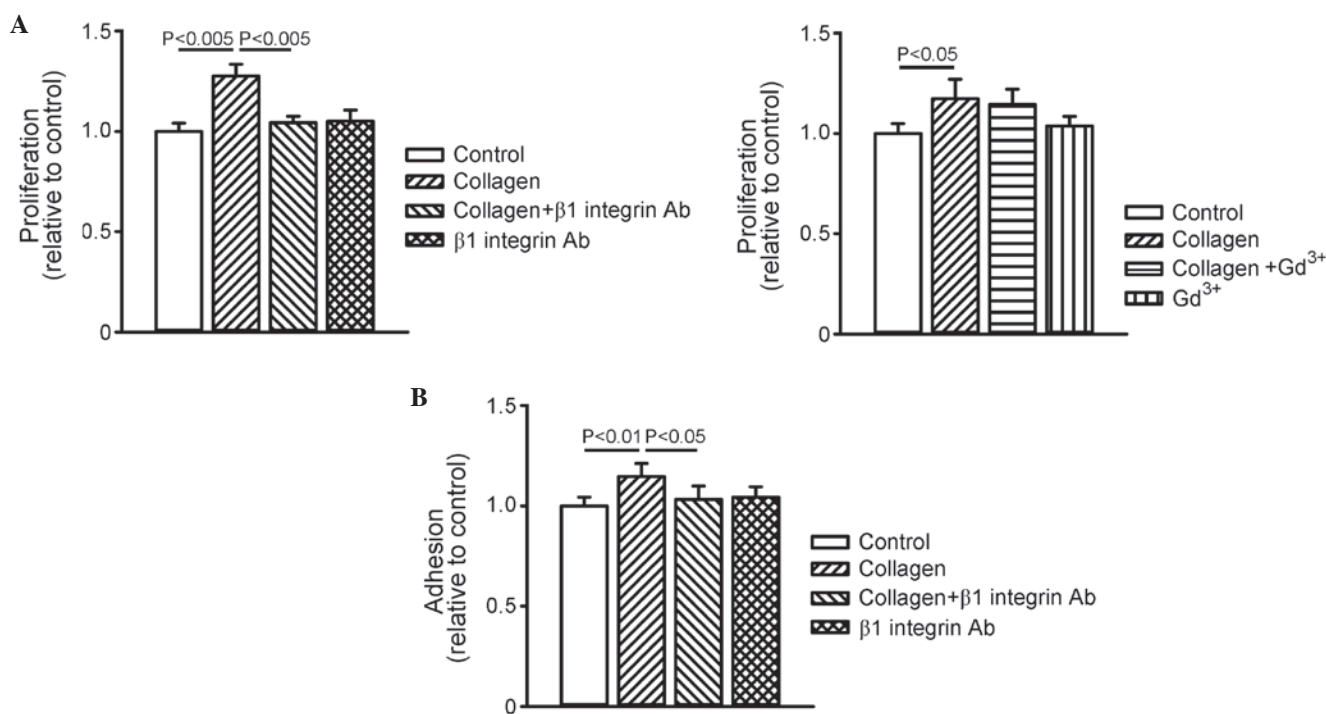


Figure 4. Effects of collagen, $\beta 1$ integrin Ab and Gd^{3+} on cell proliferation and adhesion. (A) Cell proliferation was assessed by BrdU absorbance at 450 nm. The graph on the left shows data ($n=9$) of cell proliferation in the control, collagen ($10 \mu g/ml$)-treated, collagen ($10 \mu g/ml$) with $\beta 1$ integrin Ab ($10 \mu g/ml$)-treated, and $\beta 1$ integrin Ab ($10 \mu g/ml$)-treated groups. The graph on the right shows data ($n=9$) of cell proliferation in the control, collagen ($10 \mu g/ml$)-treated, collagen with Gd^{3+} ($50 \mu M$)-treated, and Gd^{3+} ($50 \mu M$)-treated groups. (B) Cell adhesion was assessed by crystal violet absorbance at 540 nm in the control, collagen ($10 \mu g/ml$)-treated, collagen ($10 \mu g/ml$) with $\beta 1$ integrin Ab ($10 \mu g/ml$)-treated, and $\beta 1$ integrin Ab ($10 \mu g/ml$)-treated groups. Data are presented as the mean \pm standard error of the mean ($n=9$). Gd^{3+} , gadolinium; TGF β R, transforming growth factor β receptor; Ab, antibody.

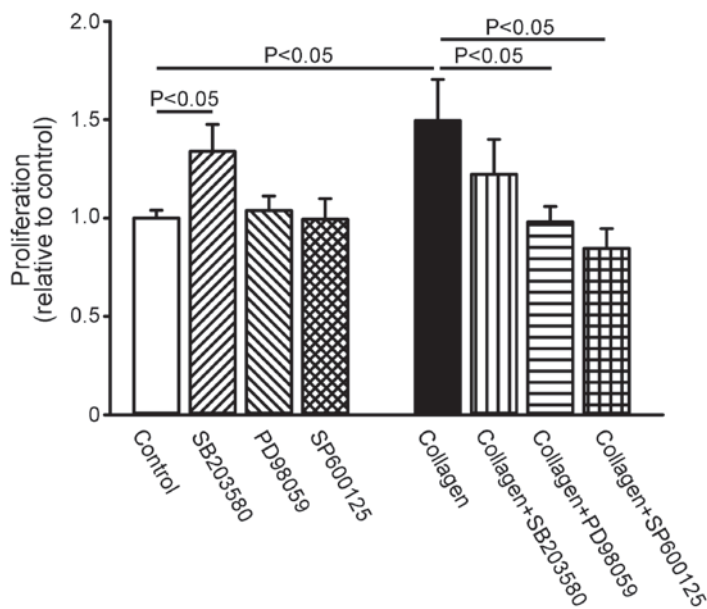


Figure 5. Effects of collagen and mitogen-activated protein kinase inhibitors on cell proliferation. Cell proliferation was assessed by BrdU absorbance at 450 nm. Cell proliferation was determined in the control, collagen ($10 \mu g/ml$)-treated, SB203580 ($3 \mu M$)-treated, PD98059 ($50 \mu M$)-treated, SP600125 ($50 \mu M$)-treated, collagen ($10 \mu g/ml$) with SB203580 ($3 \mu M$)-treated, collagen ($10 \mu g/ml$) with PD98059 ($50 \mu M$)-treated, and collagen ($10 \mu g/ml$) with SP600125 ($50 \mu M$)-treated groups. Data are presented as the mean \pm standard error of the mean ($n=9$).

Discussion

The present study is the first, to the best of our knowledge, to demonstrate that collagen directly modulated the expression of TGF- β receptors and their downstream signaling molecules.

TGF- β is an important cytokine in the fibrotic responses of several organs, including the heart (6,7). The present study demonstrated that collagen accumulation during fibrosis can downregulate the expression of TGF- β receptors. TGF- β enhances the production of the ECM from cardiac fibroblasts,

which can induce fibrosis (6,32). Although the underlying mechanisms remain to be fully elucidated, sustained TGF- β exposure has been shown to suppress the expression of TGF- β receptors (33). Therefore, an increase or decrease of collagen leads to the downregulation or upregulation of TGF- β receptor signaling, which may, at least in part, result in the known feedback loop between TGF- β and TGF- β receptors. In addition, the ECM has been reported to modulate MAPKs in cell function and differentiation (28,34,35). Collagen was found to increase the phosphorylation of p38 and ERK1/2 in the present study, which is compatible with previous findings in cardiac remodeling (36,37).

The present study found that the collagen-induced downregulation of TGF β RII was inhibited by the integrin antibody. A previous study demonstrated that the interaction of type I collagen with α 2 β 1 integrin causes downregulation of the TGF- β receptor via the activation of FAK and its diverse downstream signals, and this was found to be abrogated by treatment that inactivated FAK (35). The integrin antibody decreased the collagen-activated phosphorylation of FAK, suggesting that FAK uniquely mediates matrix-integrin interactions in cellular processes. However, integrin did not regulate the effects of collagen on TGF β R1, suggesting that collagen differentially modulates the expression of TGF β R1 and TGF β R2.

The ECM has a regulatory role in several cellular processes, including cell growth, adhesion, division and differentiation. However, whether collagen regulates cardiomyocyte proliferation and adhesion remains to be elucidated. The ECM secreted from cardiac fibroblasts has been reported to promote cardiomyocyte proliferation through β 1 integrin signaling (38). Integrins are a major class of ECM receptors for cell adhesion (20). The present study showed that collagen-binding β 1 integrin is involved in mediating cell proliferation and adhesion, which suggested that β 1 integrin may be responsible for cardiomyocyte proliferation during cardiac fibrosis. Although the inhibition of p38 enables the proliferation of cardiomyocytes (39), however, collagen-induced cell proliferation was inhibited by the inhibition of ERK in the present study. These results suggested that integrin and MAPK signaling in the cardiomyocytes may have been responsible for the effects of collagen on cardiomyocyte proliferation.

Mechanical stretch induces the release of TGF- β and is coupled with intracellular signals, which are responsible for cardiac hypertrophy (40,41). In the present study, it was found that gadolinium enhanced the downregulation of the TGF β R1, but not TGF β R2. The activation of TGF β R1 leads to the phosphorylation of Smad2 and Smad3, and regulates transcription (13,14). In the present study, collagen downregulated the phosphorylation of Smad2/3 in parallel with the expression of TGF β R1, which suggested that collagen was involved in the Smad-dependent pathway through TGF β R1. The MAPK pathway may be involved in the mechanical stretching-induced hypertrophic response (42,43). In the present study, it was found that gadolinium decreased the collagen-induced phosphorylation of p38 and ERK1/2. As gadolinium only reversed the effects of collagen on the activity of MAPKs in cells without the presence of the β 1-integrin antibody, it is possible that collagen activated the

stretch-induced MAPK signaling pathway independently of integrin-FAK signaling (44). However, collagen and gadolinium showed synergistic effects in inhibiting the expression of TGF β R1 and phosphorylation of Smad2/3. These results suggested the presence of cross-talk between collagen and mechanical stretch in the TGF β R1/Smad signaling pathways. The present study demonstrated that collagen regulated the expression of TGF- β receptors and phosphorylation of MAPKs through at least two distinct mechanisms. Collagen regulated the expression of TGF β R2 through cross-talk between integrin-FAK signaling and mechanical stretch, whereas the collagen-induced expression of TGF β R1 and activation of MAPKs were predominantly mediated by mechanical stretch. In addition, collagen may modulate the phosphorylation of JNK beyond the mechanisms identified in the present study. In the present *in vitro* study, an atrial cell line from mice was used, and these highly purified cells were placed on an artificial collagen matrix, which may or may not be reflective of human atrial cells. The findings suggested that coordinated actions occurred among the ECM, integrin and mechanical stretch in response to cardiac fibrosis.

In conclusion, the present study demonstrated that collagen was involved in altering intracellular signaling in a positive and negative manner. The observations indicated that collagen modulated the expression of TGF- β receptors and the phosphorylation of MAPKs through interactions with integrin and mechanical stretch.

Acknowledgements

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