Transforming growth factor-β1 stimulates collagen matrix remodeling through increased adhesive and contractive potential by human renal fibroblasts

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Abstract

Renal tubulointerstitial fibrosis is the common final pathway leading to end-stage renal failure. Tubulointerstitial fibrosis is characterized by fibroblast proliferation and excessive matrix accumulation. Transforming growth factor-β1 (TGF-β1) has been implicated in the development of renal fibrosis accompanied by α-smooth muscle actin (α-SMA) expression in renal fibroblasts. To investigate the molecular and cellular mechanisms involved in tubulointerstitial fibrosis, we examined the effect of TGF-β1 on collagen type I (collagen) gel contraction, an in vitro model of scar collagen remodeling. TGF-β1 enhanced collagen gel contraction by human renal fibroblasts in a dose- and time-dependent manner. Function-blocking anti-α1 or anti-α2 integrin subunit antibodies significantly suppressed TGF-β1-stimulated collagen gel contraction. Scanning electron microscopy showed that TGF-β1 enhanced the formation of the collagen fibrils by cell attachment to collagen via α1β1 and α2β1 integrins. Flow cytometry and cell adhesion analyses revealed that the stimulation of renal fibroblasts with TGF-β1 enhanced cell adhesion to collagen via the increased expression of α1 and α2 integrin subunits within collagen gels. Fibroblast migration to collagen was not up-regulated by TGF-β1. Furthermore, TGF-β1 increased the expression of a putative contractile protein, α-SMA, by human renal fibroblasts in collagen gels. These results suggest that TGF-β1 stimulates fibroblast–collagen matrix remodeling by increasing both integrin-mediated cell attachment to collagen and α-SMA expression, thereby contributing to pathological tubulointerstitial collagen matrix reorganization in renal fibrosis.
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1. Introduction

Renal tubulointerstitial fibrosis causes the progressive renal dysfunction that leads to end-stage renal failure in renal diseases. Tubulointerstitial fibrosis is characterized by renal fibroblast proliferation and excessive matrix accumulation in the renal tubulointerstitial compartment following renal injury [1–4]. Transforming growth factor-β1 (TGF-β1) is a prime fibrogenic cytokine in renal interstitial fibrosis [1,2,4]. The fibrogenic potential of TGF-β1 in renal fibrosis has been reported by several investigators [5–7]. For example, TGF-β1 increases the synthesis and reduces the degradation of extracellular matrix (ECM) protein including collagens and fibronectin in renal fibroblasts, and may thereby stimulate the renal interstitial deposition of ECM [5,6]. In addition, mice expressing the TGF-β1 transgene in the liver under the control of the murine albumin promoter developed glomerulosclerosis and tubulointerstitial fibrosis [7]. Interestingly, the subcutaneous administration of TGF-β1 to rats results in the formation of a granulation tissue which contains a large number of fibroblasts that are positive for α-smooth muscle actin (α-SMA), a myofibroblastic marker [8]. α-SMA-positive fibroblasts have been implicated in the renal interstitial fibrosis, renal dysfunction, and prognosis in IgA nephropathy [9]. These evidences suggest that TGF-

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β1 plays an important role in the differentiation of fibroblasts into myofibroblasts, which are involved in wound healing and pathological fibrocontractive diseases. However, there have been no previous studies of how TGF-β1 affects the ability of renal fibroblasts with respect to the organization of ECM protein, particularly collagen type I (collagen), a major ECM protein component in renal interstitial fibrosis.

The integrins are a large family of heterodimeric cell adhesion receptors involved in cell–cell and cell–matrix interactions [10]. They are composed of noncovalently linked α and β subunits. The integrins include all of the known cellular receptors for collagen matrix, i.e., α1β1, α2β1, α3β1, α10β1, α11β1 heterodimers [11]. Two of the best known collagen receptors are α1β1 and α2β1. α1β1 integrin is abundant on smooth muscle cells and glomerular mesangial cells, whereas α2β1 integrin is the major collagen receptor on epithelial cells and platelets. Many cell types, such as fibroblasts, osteoblasts, chondrocytes, endothelial cells, and lymphocytes concomitantly express both receptors [11]. These integrin receptors modulate critical cellular processes, including adhesion, migration, survival, ECM organization, and differentiation. These processes are physiologically related to growth, development, and tissue homeostasis, but may also be significant in pathological conditions such as renal fibrosis [12]. In fact, Roy-Chaud-
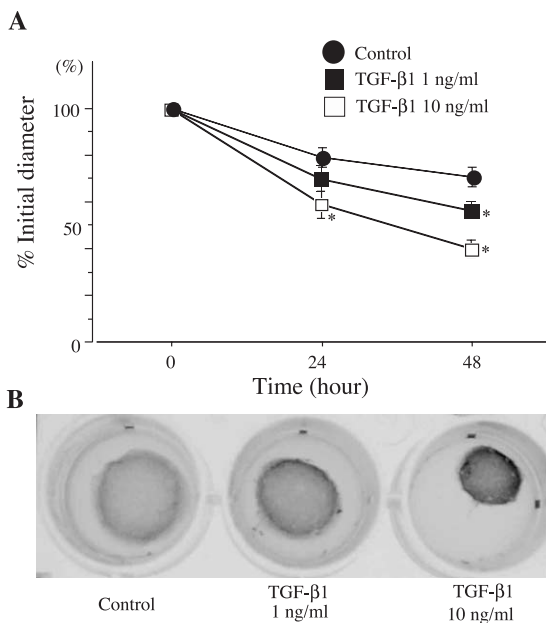

Fig. 2. Effect of anti-α1 mAb or anti-α2 mAb on collagen gel contraction by TGF-β1-treated human renal fibroblasts. The degree of TGF-β1 (1 or 10 ng/ml)-treated or untreated (Vehicle) gel contraction by human renal fibroblasts in the presence of anti-α1 mAb (20 μg/ml) (α1), anti-α2 mAb (20 μg/ml) (α2), or both anti-α1 mAb (20 μg/ml) and anti-α2 mAb (20 μg/ml) (α1 + α2) was compared to that in the presence of control mouse IgG1 (C) at 48 h. The results represent the mean ± S.D. (*P < 0.01, **P < 0.05 versus control). N.S. = not significant.

hury et al. [13] reported that the expression of α1β1 and α2β1 integrins is up-regulated in human renal interstitial fibrosis.

We hypothesized that both α1β1 and α2β1 integrins might play an important role in TGF-β1-induced pathological ECM organization by renal fibroblasts. To investigate the molecular mechanisms involved in the abnormal collagen matrix organization by renal fibroblasts, we examined the effect of TGF-β1 on collagen gel contraction, an in vitro model of collagen matrix remodeling and scar formation. The present study showed that TGF-β1 stimulates collagen gel contraction via an increase in α1β1 and α2β1 integrin-mediated cell attachment to collagen. In addition, TGF-β1 may synergistically enhance gel contraction through the increased expression of α-SMA by renal fibroblasts.

Fig. 1. Effect of TGF-β1 on collagen gel contraction by human renal fibroblasts. (A) Human renal fibroblasts cultured in collagen gels were treated with TGF-β1 (1 or 10 ng/ml) for 48 h. The degree of gel contraction by TGF-β1-treated cells was compared with that by untreated cells (Control) at 24 and 48 h. There was a significant difference between control and 10 ng/ml at 24 h. There was also a significant difference between control and 1 ng/ml, or 1 and 10 ng/ml at 48 h. The results represent the mean ± S.D. (*P < 0.01). (B) The representative photomicrographs of collagen gels at 48 h.

Table 1
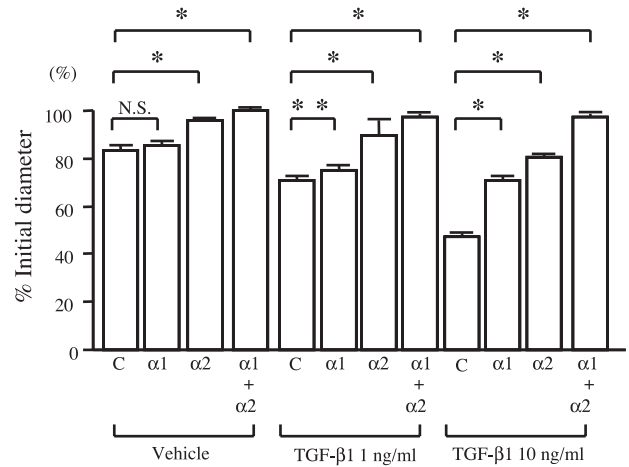Summary of the data shown in Fig. 2

	Control	Anti-α1 mAb	Anti-α2 mAb	Anti-α1 and -α2 mAb
Vehicle	83.3 ± 4.2	85.3 ± 2.1[N.S.]	95.8 ± 1.0[a]	99.7 ± 0.6[a]
TGF-β1 (1 ng/ml)	70.8 ± 1.2[b]	75.1 ± 2.1[c]	90.4 ± 6.2[a]	97.6 ± 2.1[a]
TGF-β1 (10 ng/ml)	47.5 ± 2.2[d]	70.9 ± 4.7[a]	80.8 ± 1.7[a]	97.5 ± 2.2[a]

Vehicle, in the absence of TGF-β1: Control, control antibodies (mouse IgG1). The results represent the mean ± S.D. N.S. = not significant.
  [a] P < 0.01.
  [b] P < 0.01 versus vehicle.
  [c] P < 0.05 versus control.
  [d] P < 0.01 versus TGF-β1 (1 ng/ml).

2. Materials and methods

2.1. Antibodies and reagents

Monoclonal anti-human α1 integrin subunit antibody (FB12) (anti-α1 mAb) and anti-human α2 integrin subunit antibody (P1E6) (anti-α2 mAb) were purchased from Chemicon Int. (Temecula, CA). These antibodies have been shown to block integrin adhesive function in several studies using human skin and renal fibroblasts [14–17]. They were applied at the same protein concentration (20 μg/ml) as function-blocking antibodies [14–16]. Polyclonal rabbit anti-β1 integrin subunit antibody (anti-β1 Ab) was a kind gift from Drs. K. Löster and W. Reutter (Freie Universitat Berlin, Germany). Mouse myeloma IgG1 (mIgG1) (Zymed Laboratories Inc., San Francisco, CA) and normal rabbit IgG (Rab IgG) (Zymed Laboratories Inc.) were used as control antibodies for all experiments. Fluorescein isothiocyanate (FITC)-conjugated donkey polyclonal antibody against either mouse or rabbit IgG was purchased from Jackson ImmunoResearch Laboratories Inc. (West Grove, PA). Anti-α-SMA monoclonal antibody (1A4) and anti-β-actin monoclonal antibody (AC-15) were purchased from Sigma Chemical Co. (St. Louis, MO). Horseradish peroxidase (HRP)-conjugated rabbit anti-mouse IgG (New England Biolabs, Beverly, MA) was used for Western blot analysis.

2.2. Cells and culture

Human renal interstitial fibroblast cell line (Tk188) has been established and characterized as previously described [18,19]. This cell line was obtained from the medullary part
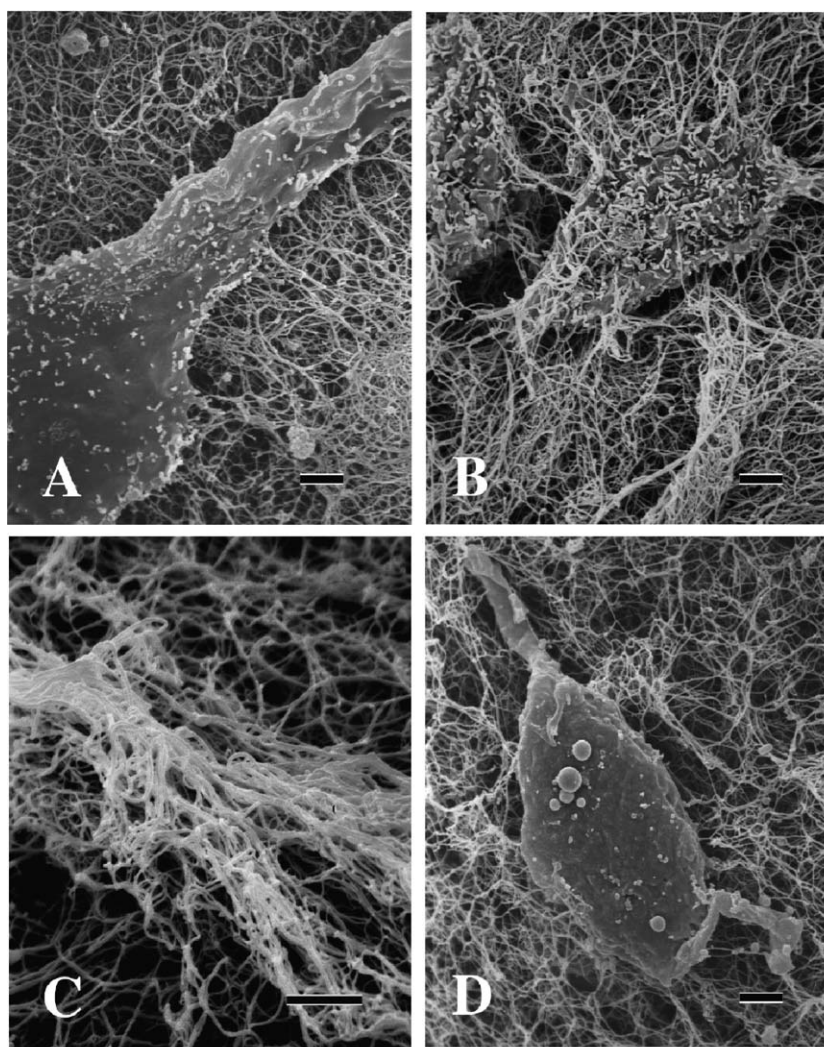

Fig. 3. Effect of anti-α1 mAb and anti-α2 mAb on the formation of collagen fibrils by TGF-β1-treated human renal fibroblasts. Renal fibroblasts and the formation of collagen fibrils in collagen gels for 48 h were examined by scanning electron microscopy. (A) Control experiments. × 42,000. (B) Renal fibroblasts treated with TGF-β1(10 ng/ml). TGF-β1 increased the number of prickles or collagen fibrils anchored to the cell surface. × 42,000. (C) The representative formation of condensed and packed collagen fibrils in collagen gels. × 96,000. (D) Renal fibroblasts treated with TGF-β1 (10 ng/ml) and anti-α1 and α2 mAbs (20 μg/ml). Anti-α1 and α2 mAbs suppressed TGF-β1-induced prickles on the cell surface and abolished the formation of condensed and packed collagen fibrils. × 42,000. Bars = 2 μm.

of a kidney biopsy with histologically proven GN and interstitial fibrosis. The use of the cells was approved by the local ethics committee and informed consent was obtained from the patient. All experiments were performed after incubation for 24 h in serum-free RPMI 1640 (Gibco BRL, Grand Island, NY) to achieve fibroblast quiescence.

2.3. Collagen gel contraction assay

Collagen gel contraction assays were performed to examine the ability of human renal fibroblasts to reorganize and contract three-dimensional collagen gels [20]. Quiescent

fibroblasts were harvested and suspended in the absence or presence of anti-α1 mAb or anti-α2 mAb at a concentration of 4×10^5 cells/ml, mixed with collagen solution [59% $1.25 \times$ RPMI 1640, 40% rat tail collagen (3.75 mg/ml), 1% 0.2N NaOH]. Final collagen concentration is 0.75 mg/ml. Collagen/cell suspensions were incubated in 12-, 24- (Costar, Cambridge, MA), or 96- (Becton-Dickinson Labware, Lincoln Park, NJ) well plates at 37 °C for 1 h to polymerize the collagen, and the gels were then gently cut away from the sides of the well and lifted off the bottom by adding serum-free RPMI 1640. The diameter of the hydrated gels was measured using an inverted microscope at 24 and 48 h. TGF-
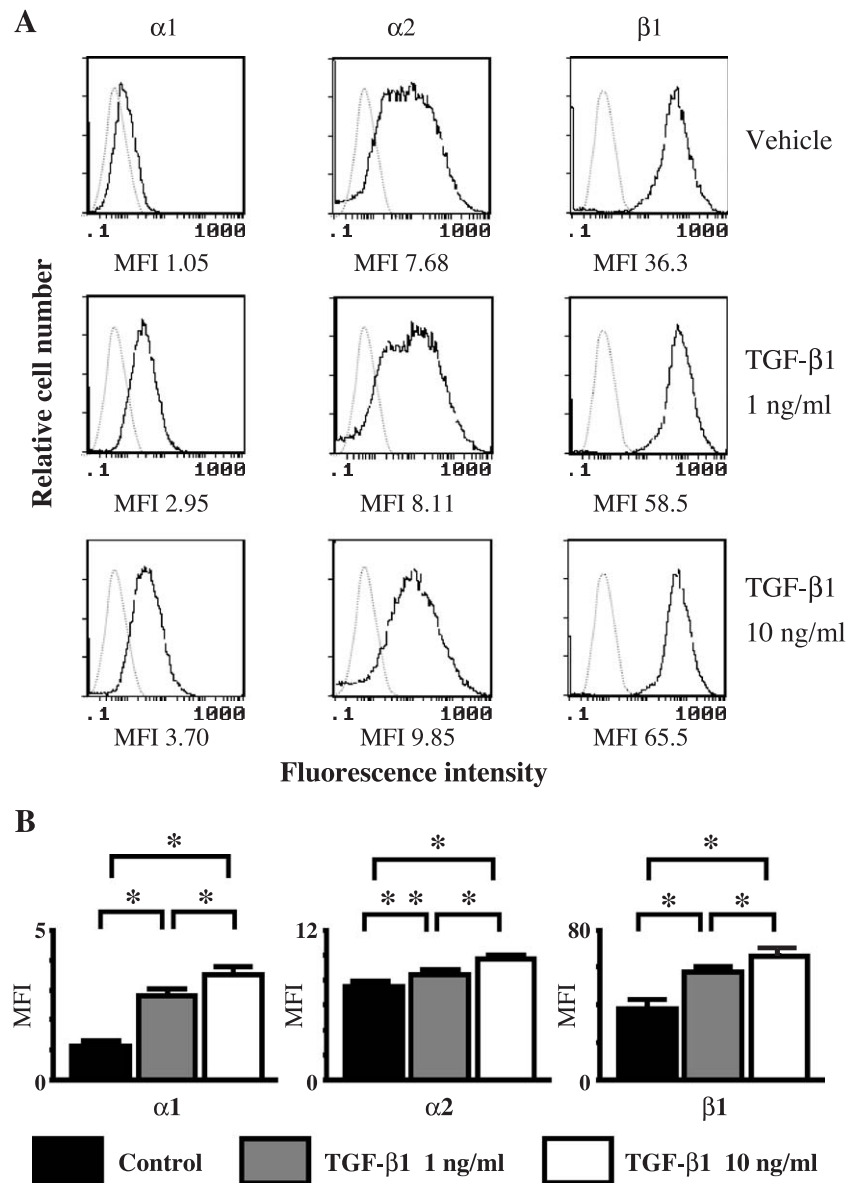

Fig. 4. Effect of TGF-β1 on the expression of α1, α2, and β1 integrins by human renal fibroblasts in collagen gels. (A) The representative data of integrin expression by flow cytometry analyses. After collagen gel-cultured fibroblasts were treated with or without TGF-β1 (1 or 10 ng/ml) for 48 h, fibroblasts were isolated and subjected to flow cytometry analysis with either anti-α1 mAb, anti-α2 mAb, or anti-β1 Ab. For background staining, the primary antibody was appropriately replaced with mouse IgG1 (mIgG1) or normal rabbit IgG (Rab IgG) (dotted lines). The mean fluorescence intensity (MFI) is shown under the histogram. The MFI of mIgG1 and Rab IgG were 0.528 and 1.13, respectively. (B) The results were shown as mean ± S.D. by analyzing MFI of integrins. (*P < 0.01, **P < 0.05). (α1) α1 integrin subunit. (α2) α2 integrin subunit. (β1) β1 integrin subunit.

β1 (1 and 10 ng/ml) was added to collagen/cell suspension before gel polymerization and when the gels were lifted.

2.4. Scanning electron microscopy

Renal fibroblasts cultured in collagen gels for 48 h were fixed with 2.5% glutaraldehyde in 0.1 M phosphate-buffered saline (PBS), pH 7.4. The fixed specimens were rinsed with the same buffer for 15 min and postfixed with 1% osmium tetroxide for 1 h. After dehydration by passage through a graded ethanol series, the specimens were infiltrated with *tert*-butyl alcohol. These specimens were dried by critical point dry methods, and coated with gold and paradium by an ion coating device (S-800, Hitachi, Japan). The specimens were then examined with scanning electron microscope (E5150, Polaron Equipment Lim., Watford, UK) [21].

2.5. Flow cytometry analyses

Flow cytometry analyses were performed to examine the cell surface expression of the α1, α2, and β1 integrin subunits in human renal fibroblasts cultured on plastic or in collagen gels [22]. Isolated fibroblasts from either culture plates (Nunc Inc., Naperville, IL) with 0.05% trypsin and 0.02% EDTA, or digested collagen gels with 1 mg/ml collagenase type II (Sigma) were incubated with either anti-α1 mAb, anti-α2 mAb, or anti-β1 Ab, washed, and resuspended in the appropriate FITC-conjugated secondary antibodies. Regarding the artificial effect of collagenase on cell surface integrin expression during the isolation of cells from collagen gels, we confirmed that collagenase did not affect surface integrin expression by renal fibroblasts cultured on collagen-coated plates. Stained cells were analyzed for fluorescence on a FACStar Plus Flow Cytometer (Becton-Dickinson). For background staining, the primary antibody was replaced with mIgG1 or Rab IgG. Ten thousand cells were separately analyzed for FITC fluorescence to quantitate the amount of bound antibodies per cell. The fluorescence intensities of these cells were averaged. The arithmetic mean thus calculated is called the mean fluorescence intensity (MFI) [23]. Expression of integrin was shown as mean ± S.D. by analyzing MFI in three separate experiments (*$P < 0.01$, **$P < 0.05$).

2.6. Migration assay

Transwell chambers (6.5 mm; Costar) with an 8-μm pore membrane were used to assess the migratory activity of renal fibroblasts [24]. The underside of the membrane was coated overnight at 4 °C with 20 μg/ml collagen. Control wells contained no collagen. The lower chamber was rinsed twice with RPMI 1640−0.1% bovine serum albumin (BSA). Growth-arrested renal fibroblasts were detached, washed, and suspended in RPMI 1640−0.1% BSA. Cells were added to the upper chamber of the transwell at 5×10^5 cells/well. The indicated amount of TGF-β1 was included in the lower

chamber. After 6 h of incubation, renal fibroblasts on the upper surface were removed by scraping so that only cells that had migrated through the membrane remained. The membrane was fixed with methanol, stained with Diff-Quik (Baxter, McGaw Park, IL), and excised from the plastic supports and allowed to air-dry on a glass slide with Permount (Sigma). Cells were counted at ×400 magnification in 10 microscope fields per filter. Background cell numbers due to migration to wells containing no collagen were subtracted from the values that reflected collagen-dependent migration. Assays were performed in triplicate and values presented are the means ± S.D. of 30 fields from three filters.

2.7. Cell adhesion assay

Flat bottom plates (96-well) were coated overnight at 4 °C with collagen I (10 μg/ml) and then blocked with 10 mg/ml BSA for 1 h at 37 °C. Quiescent fibroblasts were pretreated with or without TGF-β1 for 48 h, and then detached, washed, and pelleted. Cells were suspended in RPMI 1640 containing 1 mM $MgCl_2$ and 1 mM $CaCl_2$, and then preincubated with anti-α1 mAb, anti-α2 mAb, or mIgG1 for 30 min at 37 °C, plated onto collagen-coated wells at 5×10^4 cells/well, and incubated at 37 °C for 50 min in the presence of anti-α1 mAb, anti-α2 mAb, or mIgG1. Cell adhesion was evaluated by staining adherent cells with 0.1% crystal violet, solubilizing cells with 0.2% Triton X-100 in PBS, and reading the absorbance at 630 nm with a Corona Microplate Reader (Corona Electric Co., Ibaragi, Japan). This quantification procedure was demonstrated to give values proportional to the number of adherent cells.
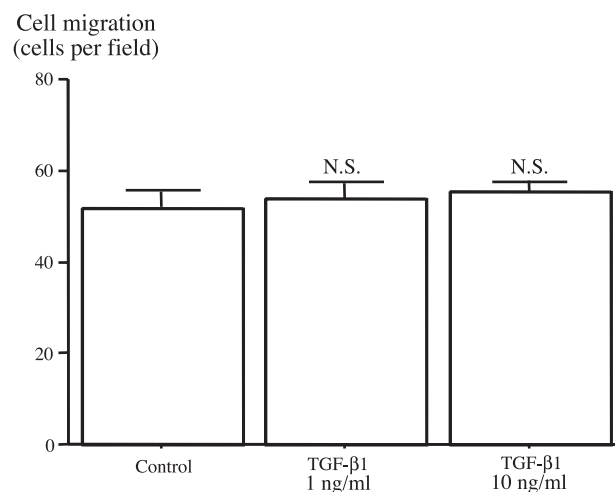

Fig. 5. Effect of TGF-β1 on migration to collagen. Human renal fibroblasts were added to the upper chamber of a transwell apparatus containing a membrane coated on the lower side with 20 μg/ml collagen. The lower chamber was supplemented with or without TGF-β1 (1 or 10 ng/ml). Cells that penetrated to the lower chamber were quantified over 6 h. Results represent the mean ± S.D. of migrated cells in 10 high-power fields. N.S. = not significant.
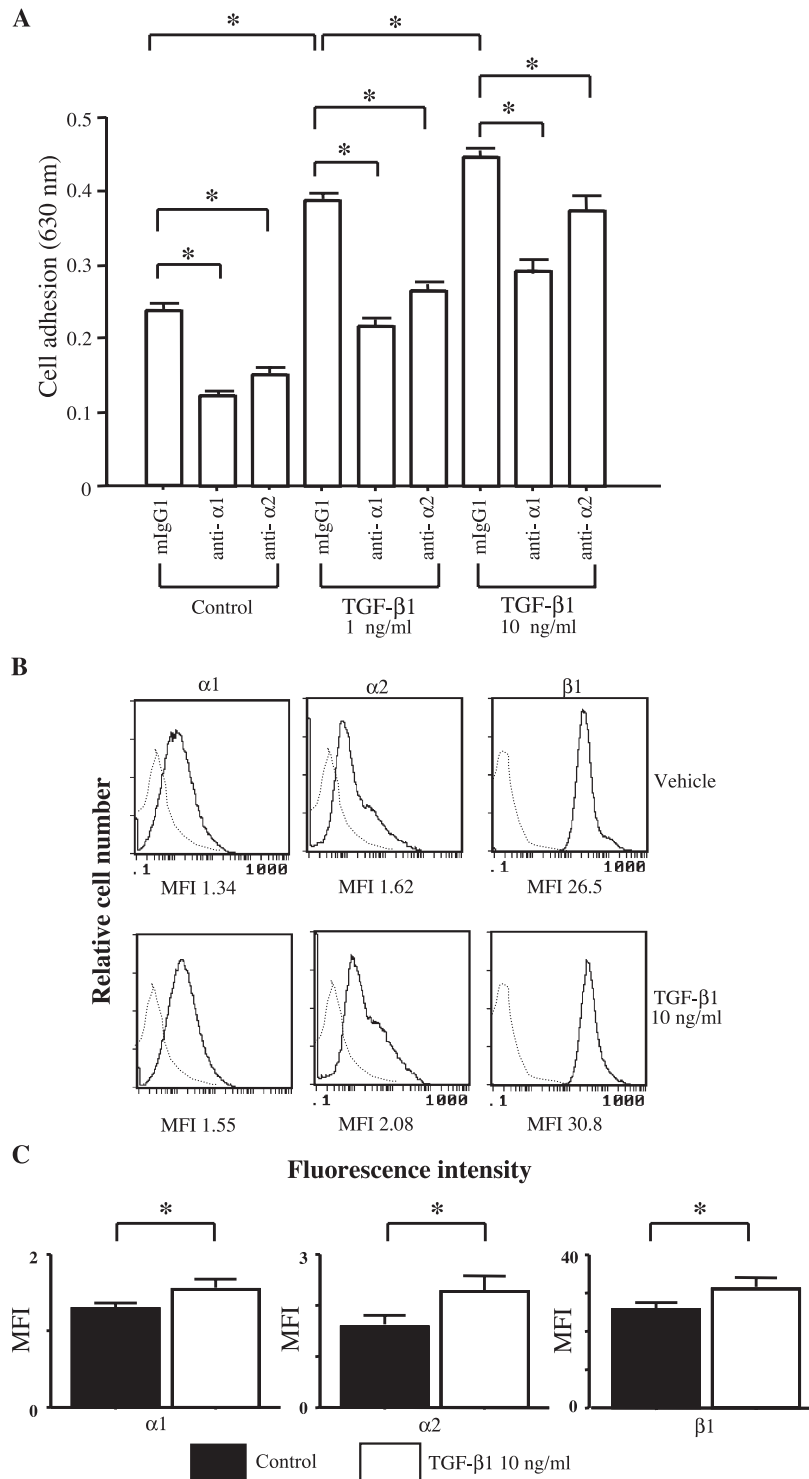
Fig. 6. (A) Effect of anti-α1 mAb or anti-α2 mAb on TGF-β1-treated fibroblast adhesion to collagen. Human renal fibroblasts were treated with TGF-β1 (1 or 10 ng/ml) or vehicle (Control) for 24 h, and then detached and preincubated with either anti-α1 mAb (20 μg/ml) (anti-α1) or anti-α2 mAb (20 μg/ml) (anti-α2) before they were added to collagen-coated wells. The degree of cell adhesion by human renal fibroblasts in the presence of anti-α1 mAb or anti-α2 mAb (20 μg/ml) was compared to that in the presence of appropriate control antibodies (mouse IgG1 (mIgG1)) at 48 h (*$P < 0.01$). (B) Effect of TGF-β1 on the expression of α1, α2, and β1 integrins by human renal fibroblasts on plastic. The representative data of integrin expression by flow cytometry analyses. After collagen gel-cultured fibroblasts were treated with or without TGF-β1 (10 ng/ml) for 48 h, fibroblasts were isolated and subjected to flow cytometry analysis with either anti-α1 mAb, anti-α2 mAb, or anti-β1 Ab. For background staining, the primary antibody was appropriately replaced with mouse IgG1 (mIgG1) or normal rabbit IgG (Rab IgG) (dotted lines). The mean fluorescence intensity (MFI) is shown under the histogram. The MFI of mIgG1 and Rab IgG were 0.412 and 0.301, respectively. (C) The results were shown as mean ± S.D. by analyzing MFI of integrins. (*$P < 0.01$). (α1) α1 integrin subunit. (α2) α2 integrin subunit. (β1) β1 integrin subunit.

2.8. Western blot analyses

Western blot analyses were performed as described previously [25]. Isolated fibroblasts from digested collagen gels were then lysed for 30 min on ice in a lysis buffer (Cell Signaling Technology, Inc., Beverly, MA). Protein samples (25 μg) were separated by 12.5% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were probed with an anti-α-SMA monoclonal antibody, and then incubated with an HRP-conjugated rabbit anti-mouse IgG. Immunoreactive proteins were detected with an enhanced chemiluminescence detection system (Amersham Corp., Arlington Heights, IL). Blots were stripped and probed with an anti-β-actin monoclonal antibody to confirm equal loading and transfer. Bands were quantitated by densitometric scanning using an LKB UltroScan XL apparatus (Pharmacia LKB Biotechnology, Uppsala, Sweden).

2.9. Statistical analyses

Results are presented as mean ± S.D. A value of $P < 0.05$ was used to determine statistical significance (t-test). Triplicate wells were analyzed for each experiment, and each experiment was performed independently a minimum of three times.

3. Results

3.1. α1β1 and α2β1 integrins mediate TGF-β1-induced collagen gel contraction by renal fibroblasts

First, we investigated whether TGF-β1 stimulates collagen matrix reorganization by renal fibroblasts using a collagen gel contraction system. As shown in Fig. 1, TGF-β1 enhanced collagen gel contraction in a dose- and time-dependent manner ($P < 0.01$, respectively). Since human renal fibroblasts express collagen-binding α1β1 and α2β1 integrins in the renal interstitium [13,15], we next examined the involvement of α1β1 and α2β1 integrins in TGF-β1-enhanced collagen gel contraction with function-blocking anti-α1 mAb and anti-α2 mAb. As shown in Fig. 2 and Table 1, collagen gel contraction was not inhibited by anti-α1 mAb under control conditions. Collagen gel contraction was suppressed by anti-α2 mAb and the combination of anti-α1 mAb and anti-α2 mAb, respectively. This suggests that collagen gel contraction is mainly mediated by α2β1 integrin under control conditions. However, TGF-β1-induced gel contraction was significantly suppressed by incubation with either anti-α1 mAb or anti-α2 mAb. The simultaneous addition of both antibodies completely inhibited collagen gel contraction. These results indicated that both integrins mediated TGF-β1-induced collagen matrix reorganization by renal fibroblasts.

3.2. TGF-β1 enhances the formation of condensed collagen fibrils by cell attachment to collagen via α1β1 and α2β1 integrins

Scanning electron microscopy revealed that TGF-β1 increased the number of prickles, which might correspond to putative interacting sites with collagen fibrils, on the cell surface, and enhanced the formation of condensed and packed collagen fibrils anchored to the cell surface as compared with control experiments (Fig. 3A and B). Fig. 3C shows the representative collagen bundles enhanced by TGF-β1. In addition, incubation with either anti-α1 mAb or anti-α2 mAb suppressed TGF-β1-induced prickles on the cell surface. Particularly, co-incubation with both antibodies abolished TGF-β1-induced prickles and formation of condensed and packed collagen fibrils (Fig. 3D).

3.3. TGF-β1 stimulates the expression of α1β1 and α2β1 integrins by renal fibroblasts within collagen gels

Previous investigations have shown that TGF-β1 enhanced the expression of β1 integrins on many types of cells, such as human osteosarcoma cells and rat mesangial cells [26,27]. However, there have been no reports on whether TGF-β1 affects the expression of α1β1 and α2β1 integrins by human renal fibroblasts within collagen gels. To examine how renal fibroblasts express α1 and α2 integrin subunits, we investigated the expression of α1, α2, and β1 integrin subunits by flow cytometry. α2 and β1 integrin subunits were strongly expressed, while α1 integrin subunit was weakly expressed on human renal fibroblasts in collagen gels. TGF-β1 (1 or 10 ng/ml) significantly enhanced the expression of α1, α2, and β1 integrin subunits in a dose-dependent manner ($P < 0.05$) (Fig. 4A and B).

3.4. TGF-β1 enhances cell adhesion, but not migration to collagen by renal fibroblasts

Cell attachment to collagen fibers and cell migration within collagen gels are critical components of collagen matrix remodeling. Therefore, we examined whether TGF-β1 could influence fibroblast adhesion and/or migration using cell adhesion and migration assays, respectively. Compared with control fibroblast migration, TGF-β1 (1 or

Table 2
Summary of the data shown in Fig. 6A

	mIgG1	anti-α1 mAb	anti-α2 mAb
Control	0.236 ± 0.011	0.119 ± 0.006[a]	0.149 ± 0.016[a]
TGF-β1 (1 ng/ml)	0.387 ± 0.010[b]	0.215 ± 0.020[a]	0.264 ± 0.017[a]
TGF-β1 (10 ng/ml)	0.446 ± 0.017[c]	0.291 ± 0.022[a]	0.369 ± 0.023[a]

mIgG1, control antibodies (mouse IgG1). The results represent the mean ± S.D.
[a] $P < 0.01$ versus mIgG1.
[b] $P < 0.01$ versus control.
[c] $P < 0.01$ versus TGF-β1 (1 ng/ml).

10 ng/ml) did not influence fibroblast migration to collagen, suggesting that migration activity is not directly involved in TGF-β1-induced gel contraction (Fig. 5). In contrast, the cell adhesion assay showed that TGF-β1 stimulated renal fibroblast adhesion to collagen in a dose-dependent manner (1 or 10 ng/ml) (Fig. 6A, Table 2). In parallel to the increased adhesion to collagen, flow cytometry revealed that TGF-β1 (10 ng/ml) significantly enhanced the expression of α1, α2, and β1 integrin subunits by renal fibroblasts cultured on plastic under the same conditions ($P < 0.01$) (Fig. 6B and C). Integrin-function-blocking experiments showed that TGF-β1-induced fibroblast adhesion to collagen is significantly suppressed by incubation with either anti-α1 mAb or anti-α2 mAb, indicating that TGF-β1-stimulated fibroblast adhesion to collagen is mediated by the increased expression of α1β1 and α2β1 integrins on renal fibroblasts (Fig. 6A, Table 2). Blocking of either α1β1 or α2β1 integrin could not completely abolish cell adhesion to collagen under TGF-β1-stimulation, suggesting that other adhesive receptors may partially play a role in cell adhesion under TGF-β1 stimulation. Collectively, these results suggest that TGF-β1 stimulates collagen gel contraction through increased α1β1 and α2β1 integrin-mediated cell attachment within collagen gels.
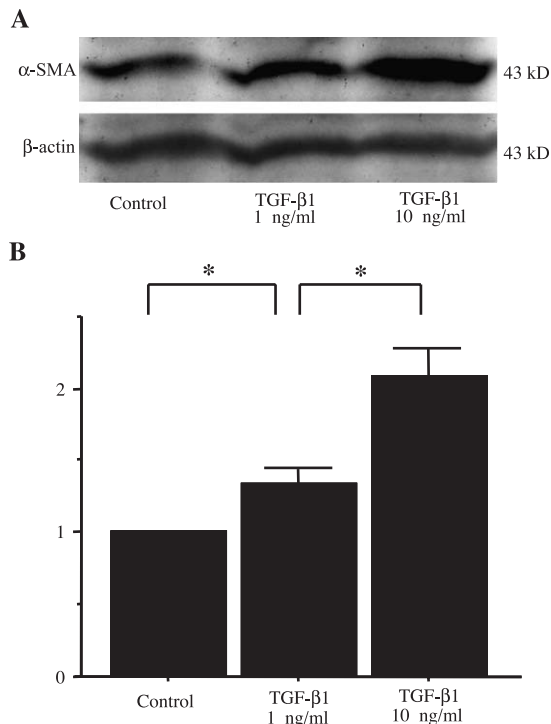

Fig. 7. Expression of α-SMA by human renal fibroblasts in collagen gels. The renal fibroblasts were incubated with TGF-β1 for 48 h. Total protein was extracted from the cultured fibroblasts. (A) The samples were adjusted to a standard content (25 μg) and analyzed by Western blotting using the anti-α-SMA monoclonal antibody. Blots were stripped and probed with an anti-β-actin monoclonal antibody to confirm equal loading and transfer. (B) The findings of densitometric analysis are analysed and the results represent the mean ± S.D. (*$P < 0.01$ versus control).

3.5. TGF-β1 stimulates the expression of α-SMA by renal fibroblasts within collagen gels

The expression of α-SMA was enhanced in the area of renal interstitial fibrosis in human diseased kidney [9]. TGF-β1 has been implicated in the activation of myofibroblasts with enhanced α-SMA expression in vivo and in vitro [8]. To clarify whether α-SMA expression is associated with TGF-β1-stimulated collagen reorganization by renal fibroblasts, the expression level of renal fibroblast α-SMA in collagen gels was investigated by Western blotting. TGF-β1 induced 1.3-fold and 2.1-fold increases at 1 and 10 ng/ml, respectively, indicating that TGF-β1 induces α-SMA protein by renal fibroblasts in collagen gels (Fig. 7).

4. Discussion

To the best of our knowledge, this is the first demonstration that TGF-β1 stimulates collagen matrix remodeling by renal fibroblasts. TGF-β1 increased the potential for fibroblasts to adhere to collagen via the increased expression of α1β1 and α2β1 integrins on the cell surface. Additionally, TGF-β1 enhanced the expression of a putative contractile protein, α-SMA, by fibroblasts within collagen gels, suggesting that TGF-β1 might influence intracellular tension leading to cell construction through myofibroblast differentiation. The three-dimensional collagen gel culture system used here has been considered to be an in vitro assay model of collagen matrix remodeling, similar to wound healing, fibrosis, and contraction scarring [28]. In this system, collagen fibers are reconstituted into thick bundles via adhesion and migration toward collagen fibers, and cell contraction during the course of gel contraction [24,25,28]. These cell behaviors appear to be essential for the fibroblast-induced irreversible, massive collagen bundle formation observed in progressive interstitial fibrosis. Considerable evidence indicates that TGF-β1 is a representative fibrogenic factor for the development of pathological interstitial ECM remodeling following renal injury [4,29,30]. Therefore, our present results suggest that the effects of TGF-β1 on integrin and α-SMA expression in renal fibroblasts are involved in TGF-β1-induced interstitial collagen matrix reorganization in diseased kidney. Recently, Roy-Chaudhury et al. [13] reported that the expression of the α1 and α2 integrin subunits was increased within the area of interstitial fibrosis in human renal diseases. Many cell culture studies using various cell types such as human osteogenic cells, skin and gingiva fibroblasts have demonstrated that α2β1 integrin is a critical collagen receptor for collagen gel contraction, and TGF-β1 enhanced gel contraction by increasing α2β1 integrin expression [16,26,31,32]. Similarly, Kelynack et al. [15] showed that collagen fibril rearrangement by human renal fibroblasts in vitro appears to be an integrin-mediated process involving the α2β1 integrin. However, we found here that human renal fibroblasts expressed α1 and

α2 integrin subunits on their surfaces within collagen gels, although expression level of α2 integrin subunit is high, while that of α1 integrin subunit is relative low. Of note, preincubation with anti-α1 mAb suppressed TGF-β1-induced gel contraction but not untreated gel contraction, suggesting that α1 integrin subunit may be an important molecule in TGF-β1-induced pathological settings such as renal interstitial fibrosis. Similarly, we have shown that α1β1 integrin is a major collagen receptor expressed on mesangial cells (MCs) for abnormal mesangial ECM remodeling in diseased glomeruli [33]. TGF-β1 stimulated α1β1 integrin-mediated MC adhesion but not migration to collagen, and thereby enhanced collagen gel contraction. Taken together, these parallel TGF-β1 action on ECM remodeling by MC and renal fibroblast may play a major role in ECM remodeling involved in the progression of kidney diseases. The difference in the expression of collagen-binding integrin subunits in human renal fibroblasts and mesangial cells might reflect the distinct functional roles of these integrins in fibrotic processes, since it has been reported that α1β1 integrin mediates the signals, including the down-regulation of collagen gene expression, whereas α2β1 integrin mediates the induction of matrix metalloproteinase-1 [34]. Further study is required to clarify the molecular mechanisms involved in α1β1 and α2β1 integrin-mediated collagen matrix remodeling by renal fibroblasts.

In addition to being a well-accepted marker for myofibroblast differentiation, α-SMA has also been suggested to play a role in the production of contractile force during wound healing and fibrocontractile disease [35]. Hinz et al. [36] showed that the enhancement of collagen gel contraction by 3T3 fibroblasts transfected with α-SMA cDNA was accompanied by increased α-SMA expression and cell contraction compared with nontransfected cells. In the present study, TGF-β1 induced α-SMA expression by renal fibroblasts in collagen gels, suggesting that TGF-β1 enhances collagen gel contraction via increased fibroblast contraction. Recent studies also showed that gel contraction by cardiac fibroblasts is stimulated by TGF-β1 [37]. This stimulation of gel contraction was accompanied by induction of α-SMA. Thus, the increase of cell cytoskeleton, such as α-SMA, as well as enhanced integrin-mediated cell binding to collagen fibrils might play a role in TGF-β1-induced collagen matrix reorganization by fibroblasts. Interestingly, we previously showed that the overexpression of α1 integrin subunit in rat mesangial cells is accompanied by the increased expression of α-SMA and enhanced collagen gel contraction [24]. Furthermore, several recent studies have indicated that the α1 integrin subunit plays a major role in the contractile capacity of several cell lines including rat cardiac fibroblasts and mesangial cells, suggesting that there is a close link between α1 integrin and α-SMA expression [20,22,38,39].

In conclusion, we have demonstrated that TGF-β1 stimulates renal fibroblast–collagen matrix reorganization through an increase in cell activity regarding integrin-mediated adhesion to collagen and contraction. Considering that TGF-β1 is a representative fibrogenic factor that induces renal interstitial fibrosis, the integrin is its downstream player for the substantial assembly of interstitial collagen matrix. Therefore, strategies that target the collagen-binding integrin expressed on renal fibroblasts may permit the development of new therapeutic methods for the inhibition of progressive renal fibrosis.

Acknowledgements

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