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TGF-β1-induced PINCH-1-ILK-α-parvin complex formation regulates mesangial cell proliferation and hypertrophy

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Abbreviations: GMCs, glomerular mesangial cells; ILK, integrin-linked kinase; PIP, PINCH-1-ILK-α-parvin; TBS, Tris-buffered saline

Abstract

TGF-β1-induced glomerular mesangial cell (GMC) injury is a prominent characteristic of renal pathology in several kidney diseases, and a ternary protein complex consisting of PINCH-1, integrin-linked kinase (ILK) and α-parvin plays a pivotal role in the regulation of cell behavior such as cell proliferation and hypertrophy. We report here that PINCH-1-ILK-α-parvin (PIP) complex regulates the TGF-β1-induced cell proliferation and hypertrophy in cultured rat GMCs. When GMCs were treated with TGF-β1 for 1, 2 and 3 days, the PIP complex formation was up-regulated after 1 day, but it was down-regulated on day 2. Cell numbers were significantly elevated on day 2, but dramatically decreased on day 3. In contrast, a significant increase in cellular protein contents was observed 3 days after TGF-β1-treatment. TGF-β1 induced early increase of caspase-3 activity. In GMCs incubated with TGF-β1 for 2 days, cytosolic expression of $p27^{Kip1}$ was dramatically reduced, but its nuclear expression was remarkably elevated. A significantly decreased expression of phospho-Akt (Ser 473) was observed in the cells treated with TGF-β1 for 1 day. TGF-β1 induced early increase of phospho-$p27^{Kip1}$ (Thr 157) expression with subsequent decrease, and similar responses to TGF-β1 were observed in the p38 phosphorylation (Thr 180/Thr 182). Taken together, TGF-β1 differently regulates the PIP complex formation of GMCs in an incubation period-dependant fashion. The TGF-β1-induced up- and down-regulation of the PIP complex formation likely contributes to the pleiotropic effects of TGF-β1 on mesangial cell proliferation and hypertrophy through cellular localization of $p27^{Kip1}$ and alteration of Akt and p38 phosphorylation. TGF-β1-induced alteration of the PIP complex formation may be importantly implicated in the development and progression of glomerular failure shown in several kidney diseases.

Keywords: α-parvin protein; apoptosis; cell proliferation; hypertrophy; integrin-linked kinase; LIMS1 protein, human; mesangial cells; rat; transforming growth factor β1

Introduction

Glomerular diseases are a leading cause of chronic and end-stage kidney failure worldwide, and an array of glomerular diseases is distinguished by glomerular mesangial cell (GMC) injury including membranoproliferative glomerulonephritis, IgA nephropathy and diabetic nephropathy. Typical fates of GMCs occurring in pathophysiological state include mesangial matrix deposition, cell proliferation and hypertrophy and apoptosis, which closely correlate with deterioration of renal function and therefore have long been considered an important factor in progressive renal failure (Mason and Wahab, 2003). Additionally, glomerular hypertrophy is one of the cellular pathological characteristics of several kidney diseases including diabetic nephropathy, and this structural alteration is principally due to out-growth of GMCs (Bak *et al*., 2000; Drummond and Mauer, 2002).

It is well known that TGF-β1 plays crucial roles in the development and progression of kidney diseases. TGF-β1 is elevated in human glomeruli

with diabetic nephropathy and focal segmental glomerulosclerosis (Mason and Wahab, 2003; Schnaper *et al*., 2003; Schrijvers *et al*., 2004) and elicits diverse effects on glomerular cells including stimulation of mesangial matrix expansion and promotion of podocyte detachment and apoptosis (Phillips *et al*., 1999; Bottinger and Bitzer, 2002; Wolf *et al*., 2005). TGF-β1 is also known to be important regulators of mesangial cell proliferation and hypertrophy under pathological condition with glomerular injury. Thus, it is considerable that TGF-β1 mediates virtually all of the pathological changes of glomerular diseases including diabetic glomerulopathy.

Cell-extracellular matrix adhesion is a key determinant of cell survival and mediated by network of transmembrane adhesion receptors such as integrins. Integrin-extracellular matrix interaction mediates the cell signals that regulate the differentiation, proliferation and survival of cells. Integrin-linked kinase (ILK), an ankyrin repeat-containing serine-threonine protein kinase, interacts with the cytoplasmic domains of β-integrins and numerous cytoskeletal-associated proteins including PINCH-1 and α-parvin. ILK interacts with PINCH-1 and α-parvin through two direct interactions, one mediated by the PINCH-1 N-terminal LIM 1 domain and the ILK N-terminal ankyrin repeat domain and the other mediated by the ILK C-terminal domain and the α-parvin calponin homology 2 domain, resulted in the formation of PINCH-1-ILK-α-parvin (PIP) complex (Tu *et al*., 2001; Zhang *et al*., 2002), which is found in several different types of mammalian cells. Guo and Wu (2002) reported that ILK forms a complex with PINCH-1 and α-parvin in cultured GMCs, and this protein (PIP) complex plays a crucial role in the regulation of mesangial cell proliferation and fibronectin matrix deposition. The PIP complex is also formed in ventricular myocytes and implicated in mediating integrin-signaling pathway which regulates cardiac myocyte hypertrophy and resistance to apoptosis (Chen *et al*., 2005). Thus, it is considered that the PIP complex serves as an important transducer of extracellular signals to control many aspects of cell morphology and behavior.

Recently, we reported that TGF-β1 induces podocyte apoptosis through the disruption of the PIP complex, but promotes the PIP complex formation in GMCs (Jung *et al*., 2007), indicating that TGF-β1-induced alteration of PIP complex formation is cell type-specific in glomerular cells and may be critically related to the cell type-specific behavior of GMCs and podocytes occurring in progressive glomerular failure, because increased TGF-β1 production in glomeruli with diabetic nephropathy induces hypertrophy and apoptosis in GMCs and podocytes, respectively. This study examined whether TGF-β1-induced alteration of the PIP complex is implicated in the development of cell proliferation and hypertrophy in primarily cultured rat GMCs, which are prominent characteristics of mesangial cell pathology in progressive glomerular diseases.

Materials and Methods

Materials and reagents

Cell culture media, RPMI 1640, and FBS were purchased from Gibco BRL (Gaithersburg, MD). Human TGF-β1 was from Chemicon (Temecula, CA). Mouse monoclonal anti-ILK antibody (clone 65.1), anti-α-parvin antibodies (clones 1D4.5 and 3B5) and rabbit polyclonal anti-PINCH-1 antibody were previously described (Tu *et al*., 2001; Jung *et al*., 2007). Antibodies recognizing p38, phospho-p38 (Thr 180/Tyr 182), Akt, phospho-Akt (Ser 473), $p27^{Kip1}$, and phospho-$p27^{Kip1}$ (Thr 157) were purchased from Cell Signaling Technology (Beverly, MA). All other chemicals and reagents were of the highest grade from commercial sources.

Mesangial cell culture

Primarily cultured GMCs were obtained from a culture of glomeruli isolated from the male Sprague-Dawley rats as described previously (Rho *et al*., 2004). In brief, the isolated glomeruli were cultured in RPMI 1640 media containing 15 mM HEPES, 20 mM $NaHCO_3$, 20% heat-inactivated FBS, 100 U/ml penicillin, 100 μg/ml streptomycin and 1 × insulin-transferrin-selenium A solution supplement (Life Technologies, Grand Island, NY) under 95% air/5% CO_2 condition. After four passages, cells were cultured with 10% FBS-contained RPMI 1640 media. The cultured cells were identified as GMCs by their typical morphology and immunohistochemical characters as described previously (Harper *et al*., 1984). For experiments, at 2 days after plating GMCs, cells were cultured with 1% FBS-contained RPMI 1640 media with or without TGF-β1 (2 ng/ml) for 1, 2 and 3 days. The cells were then harvested and analyzed by cell proliferation and hypertrophy, immunoprecipitation and Western blotting as described below.

Cell proliferation and hypertrophy assay

Cell proliferation and hypertrophy were measured by the previous method (Wahab *et al*., 2002). At

the end of stimulation period, GMCs were washed three times with ice-cold PBS (pH 7.4) and collected using 0.25% trypsin and 0.5% EDTA, followed by centrifuging at 1,000 *g* for 5 min. Cell pellets were resuspended with 1 ml of ice-cold PBS, and cell numbers were counted using an improved Neubauer hemocytometer. Equal numbers of cells were lysed in RIPA buffer (0.1% SDS, 0.5% sodium deoxycholate and 1.0% Nonidet P-40 in PBS). Total protein content was measured using the BCA-200 protein assay kit (Pierce, Rockford, IL). Total protein content was expressed as micrograms of protein per 10^4 cells. Experiments were independently performed three times.

Immunoprecipitation

The PIP complex formation was analyzed by immunoprecipitation and Western blotting as we described previously (Yang *et al*., 2005; Jung *et al*., 2007). Briefly, GMCs were lysed with the lysis buffer (1.0% Triton X-100, 150 mM NaCl, 5.0 mM EDTA, 2.0 mM Na_3VO_4, 2.5 mM Na_4PO_7, 100 mM NaF, protease inhibitors, 50 mM Tris-HCl, pH 7.4). The cell lysates (500 μg) were mixed with 10 μg of mouse monoclonal anti-α-parvin antibody (clone 1D4). The samples were incubated for overnight at 4°C and mixed with UltraLink Immobilized protein A/G (Pierce) and then incubated for additional 2 h. The beads contained in samples were washed five times, and proteins were released from the beads by boiling in SDS-PAGE sample buffer for 5 min. The samples were analyzed by Western blotting with mouse monoclonal anti-α-parvin antibody (clone 3B5), mouse monoclonal anti-ILK antibody (clone 65.1) and rabbit polyclonal anti-PINCH-1 antibody.

Cytosolic and nuclear protein extraction

Extraction of cytosolic and nuclear proteins was performed as previously described by Caruccio and Banerjee (1999) with slight modification. Briefly, cell pellets in Eppendorf tubes were resuspended in about 150 μl of Buffer A [10 mM HEPES (pH 7.9), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM DTT and 0.25 mM PMSF] and left on ice for 10 min with occasional tapping. After mixing by vortex for 30 s, 30 μl of cell extracts were taken for measurement of total protein amount. The nuclei were collected by centrifuging at 14,000 rpm for 10 s at 4°C. The cytosolic supernatant fractions were removed to another Eppendorf tubes and "snap-frozen" on dry ice. The pelleted nuclei were resuspended with into Buffer B [20 mM HEPES (pH 7.9), 0.4 M NaCl, 1.5 mM $MgCl_2$, 0.5 mM EDTA, 20% glycerol, 0.5 mM DTT, 0.25 mM PMSF] on ice for 30 min with periodic mixing by tapping to extract the nuclear proteins. The nuclear fractions were then centrifuged at 14,000 rpm for 5 min at 4°C, and the supernatant fractions were collected to analyze nuclear proteins by Western blotting.

SDS-PAGE and Western blot analysis

After GMCs were washed three times with ice-cold PBS (pH 7.4) and collected using 0.25% trypsin and 0.5% EDTA, followed by centrifuging at 1,000 *g* for 5 min. The cells were lysed in the Lysis buffer (20 mM Tris/HCl, 1% SDS, pH 7.4) and kept on ice for 30 min. After adding SDS-PAGE sample buffer, samples were boiled for 5 min and resolved on 10% gel by SDS-PAGE. Proteins were transferred onto a polyvinylidene difluoride membrane (Immobilin-P, Millipore, Bedford, UK) using a BioRad (Hercules, CA) transfer apparatus. Blots were incubated in blocking buffer containing 1 × Tris-buffered saline (TBS), 0.1% Tewwn-20 with 5% (w/v) nonfat dry milk for 1 h. Immunodetection was performed by incubating the blots in primary antibody at the appropriate dilution in antibody reaction buffer (1 × TBS, 0.1% Tween-20 and 10% BSA) for overnight at 4°C. Blots were then washed three times with washing buffer (1× TBS and 0.1% Tween-20) and incubated with HRP-conjugated secondary antibody for 1 h at room temperature. Bound antibodies were visualized using the enhanced chemiluminescence reagent Luminol (Pierce, Rockford, IL). Prestained molecular weight markers (BioRad) were used to confirm protein migration.

Caspase-3 assay

As we described previously (Jung *et al*., 2007), to analyze cellular apoptosis, caspase-3 activity was measured using fluorogenic caspase-3 substrate VII (Ac-DEVD-AFC, Calbiochem, San Diego, CA) following the manufacturer's protocol. Briefly, GMCs were treated with 2 ng/ml of TGF-β1 for 1, 2 and 3 days and washed three times with ice-cold PBS. The cells were lysed with the lysis buffer (50 mM HEPES, 100 mM NaCl, 0.1% CHAPS, 1 mM DTT and 0.1 mM EDTA, pH 7.4). The cell lysates (triplicate per sample) were mixed with the fluorogenic caspase-3 substrate VII and fluorescence (excitation at 400 nm and emission at 505 nm) and measured using a TECAN GENios PRO fluorescence microplate reader. Results were expressed as % changes of control, and experiments were independently performed three times.

Statistical analysis

All statistical analyses were performed using the Microsoft Excel data analysis program for Kruskal-Wallis test with Dunn's multiple comparison test or Statview ver 6.0 for analysis of variance with Bonferroni's test and Mann-Whitney rank sum test. Values were expressed as mean ± S.E.

Results

TGF-β1-induced PINCH-1-ILK-α-parvin complex formation

Using anti-α-parvin monoclonal antibody (clone 1D4.5), α-parvin was immunoprecipitated after GMCs were incubated with or without TGF-β1 (2 ng/ml) for 1, 2 and 3 days, and Western blotting analysis was carried out using anti-α-parvin antibody (clone 3B5). A similar amount of α-parvin was immunoprecipitated from the GMCs treated with and without TGF-β1 (Figure 1B, anti-α-parvin). Western blotting using the same samples with an anti-PINCH-1 antibody showed that the amount of PINCH-1 co-immunoprecipitated with α-parvin increased in the cells treated with TGF-β1 for 1 day, but significantly decreased when cells were treated with TGF-β1 for 2 days (Figure 1B, anti-PINCH-1). The amount of ILK that co-immunoprecipitated with α-parvin was also substantially changed in response to TGF-β1 (Figure 1B, anti-ILK). In contrast, total protein levels of PINCH-1, ILK and α-parvin were not significantly changed (Figure 1A). These results indicate that the treatment of GMCs with TGF-β1 changes the complex formation of PINCH-1 and ILK with α-parvin in a time-dependent fashion.

TGF-β1-induced cell proliferation and hypertrophy

To evaluate whether the PIP complex plays a role in the regulation of mesangial cell proliferation and hypertrophy, this study measured cell numbers and protein contents from GMCs treated with or without TGF-β1 (2 ng/ml) for 1, 2 and 3 days. As shown in Figure 2A, the exposure of cells to TGF-β1 for 2 days resulted in a significant increase in cell numbers, but these were dramatically reduced on day 3. There was no significant difference in cell numbers between the cells treated with and without TGF-β1 for 1 day. Interestingly, a significant increase in cellular protein contents was only observed in the GMCs treated with TGF-β1 for 3 days (Figure 2B). Thus, TGF-β1 induces early proliferation of GMCs, followed by cell hypertrophy, which is characterized by a cellular pathological

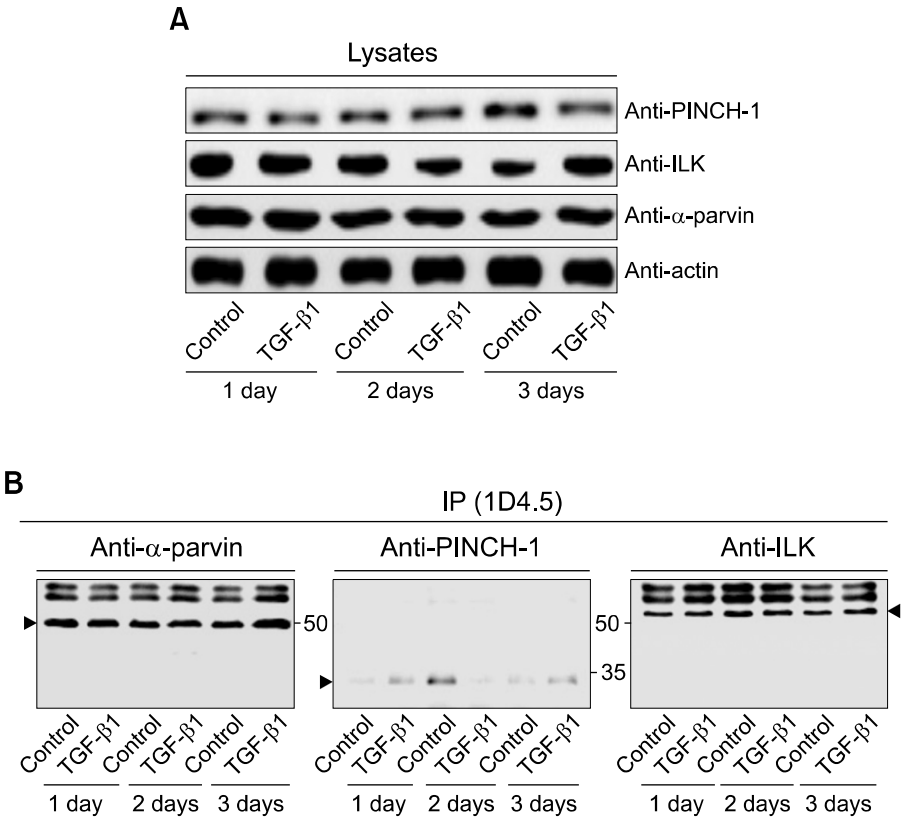

Figure 1. TGF-β1-induced alteration of the PINCH-1-ILK-α-parvin complex formation in cultured rat glomerular mesangial cells. Cells were cultured under 1% serum-contained condition with or without TGF-β1 (2 ng/ml) for 1-3 days, and immunoprecipitation was carried out with anti-α-parvin monoclonal antibody (1D4.5). Cell lysates (A) and anti-α-parvin immunoprecipitates (B) were analyzed by Western blotting with antibodies recognizing PINCH-1, ILK, α-parvin and actin. Note that the amount of ILK and PINCH-1 associated with α-parvin is remarkably changed in response to TGF-β1.

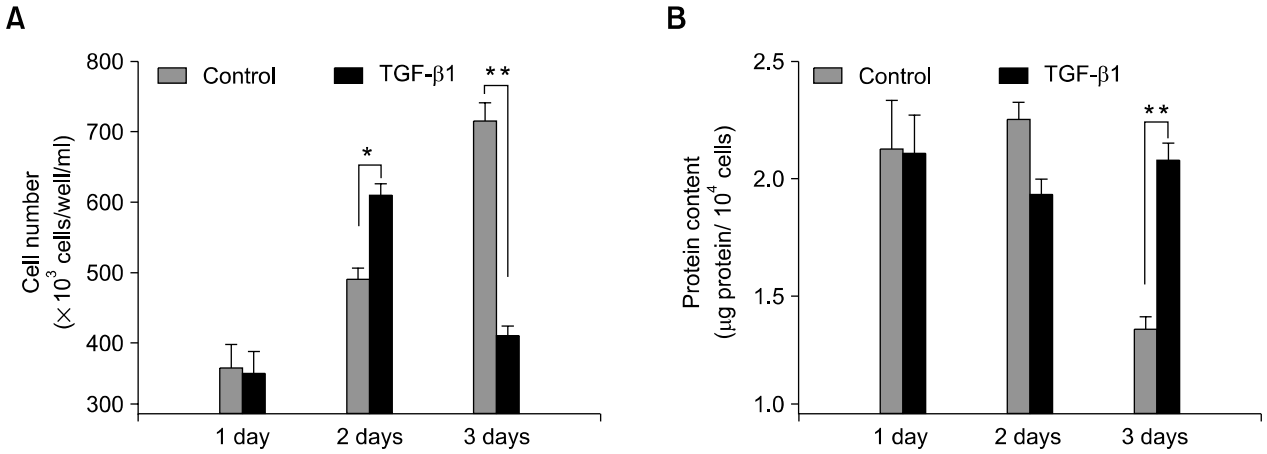

Figure 2. Changes of TGF-β1-induced proliferation and hypertrophy in cultured rat glomerular mesangial cells. Cells were cultured under 1% serum-contained condition with or without TGF-β1 (2 ng/ml) for 1-3 days, and cell number (A) and protein contents (B) were measured to estimate cell proliferation and hypertrophy as described in the Materials and Methods. Note that a significantly decreased proliferation and a remarkably increased hypertrophy are observed in the mesangial cells at 3 days after TGF-β1 treatment. *$P < 0.05$, **$P < 0.01$.

phenomenon in GMCs with several glomerular diseases.

TGF-β1-induced caspase-3 activity

As demonstrated in Figure 2, TGF-β1 causes very early and self-limited proliferation of GMCs with subsequent hypertrophy *in vitro*, and these cellular responses to TGF-β1 are possibly considered as a compensatory cellular mechanism, indicating that residual renal tissue in response to loss of other renal tissue undergoes hypertrophy (Hostetter, 1995). Based on these, this study investigated whether treatment of GMCs with TGF-β1 produces apoptotic cell death at early stage, and caspase-3 activity was measured at 1, 2 and 3 days after GMCs were incubated with or without TGF-β1 (2 ng/ml). As shown in Figure 3, caspase-3 activity was significantly elevated in the cells incubated with TGF-β1 for 1 and 2 days. However, there was no significant change of caspase-3 activity in the cells treated for 3 days.

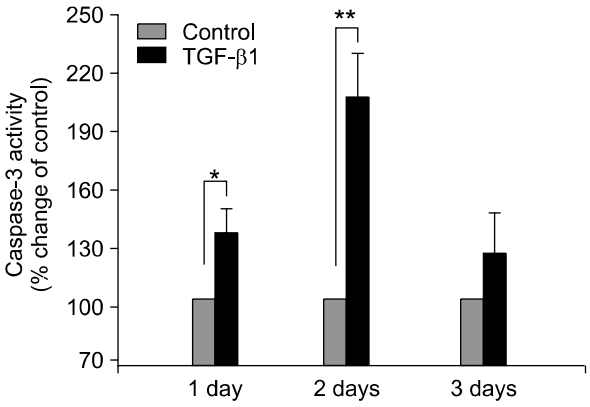

Figure 3. TGF-β1-induced change of caspase-3 activity in cultured rat glomerular mesangial cells. Cells were cultured under 1% serum-contained condition with or without TGF-β1 (2 ng/ml) for 1-3 days, and caspase-3 activity was measured to estimate apoptotic cell death as described in the Materials and Methods. Note that TGF-β1 causes a significant increase of apoptotic cell death in mesangial cells on day 2 and 3. *$P < 0.05$, **$P < 0.01$.

TGF-β1-induced intracellular localization of $p27^{Kip1}$

To delineate the contribution of $p27^{Kip1}$, a cyclin-dependent kinase inhibitor, to the TGF-β1-induced mesangial cell proliferation and hypertrophy through modifying the PIP complex formation, we examined intracellular localization of $p27^{Kip1}$ in GMCs incubated with or without TGF-β1 (2 ng/ml) for 1, 2 and 3 days. The obtained results are shown in Figure 4. Total level of $p27^{Kip1}$ expression increased in the cells treated with TGF-β1 for 1 day, but a significant decrease in its total expression was observed on 2 day. These changes was negatively correlated with expressional level of $p27^{Kip1}$ localized into nuclei, showing that nuclear localization of $p27^{Kip1}$ decreased in the cells treated with TGF-β1 for 1 day, but significantly increased on day 2. Cytosolic expression of $p27^{Kip1}$ was negatively correlated with the level of its nuclear expression. Reprobing the Western blotting with anti-actin and -tubulin antibodies showed an equal protein loading. Thus, TGF-β1 not only changes total expression of $p27^{Kip1}$, but also effectively regulate nuclear localization of $p27^{Kip1}$ in GMCs.

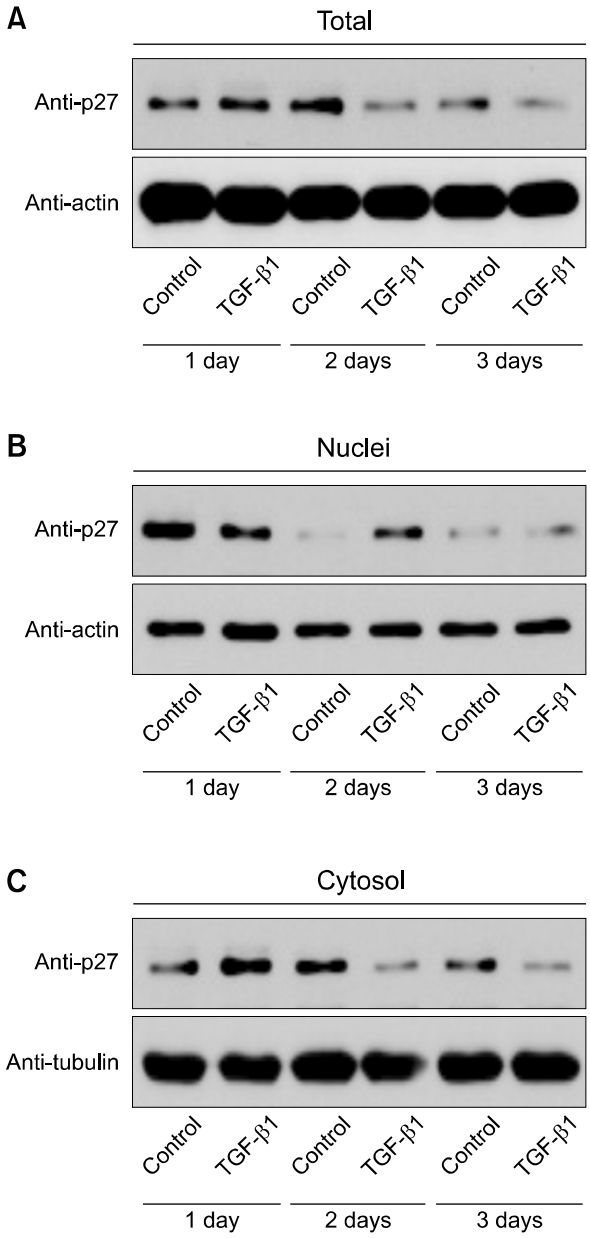

Figure 4. TGF-β1-induced intracellular localization of $p27^{Kip1}$ in cultured rat glomerular mesangial cells. Cells were cultured under 1% serum-contained condition with or without TGF-β1 (2 ng/ml) for 1-3 days. Cell lysates were fractionated into total (A), nuclei (B) and cytosol (C), and each fraction was analyzed by Western blotting with antibodies recognizing $p27^{Kip1}$, actin and tubulin. Note that total amount of $p27^{Kip1}$ is not significantly changed in response to TGF-β1, whereas $p27^{Kip1}$ expression in nuclei is negatively correlated with its cytosolic expression.

TGF-β1-induced phosphorylation of signaling proteins

This study examined the phosphorylation of Akt, $p27^{Kip1}$ and p38 MAPK in GMCs treated with TGF-β1 for 1, 2 and 3 days, and the obtained results are shown in Figure 5. Akt phosphorylation (Ser 473) was significantly decreased in the cells incubated with TGF-β1 for 1 day, after then gradually increased. Exposure of GMCs with TGF-β1 for 1 day increased the $p27^{Kip1}$ phosphorylation (Thr 157). In contrast, a significant decrease in $p27^{Kip1}$ phosphorylation was observed in the cells incubated with TGF-β1 for 2 days. Phosphorylation of p38 MAPK (Thr 180/Tyr 182) was increased in the cells incubated with TGF-β1 for 1 day, followed by a significant decrease. There was no significant difference of total amount of these signaling proteins in between the cells incubated with and without TGF-β1. Reprobing the same samples with anti-actin antibody showed an equal protein loading. Thus, it is considerable that TGF-β1 differently regulates the phosphorylation of Akt, $p27^{Kip1}$ and p38 MAPK in a time-dependent manner.

Discussion

TGF-β1 is a key mediator of mesangial cell pathology in the progression of glomerular failure, and it has been more challenging to evaluate the molecular pathomechanism implicated in the progression of glomerular failure. This study demonstrates that treatment of cultured rat GMCs with TGF-β1 changes the PIP complex formation in a time-dependent manner, and this molecular event is likely implicated in the regulation of TGF-β1-induced mesangial cell proliferation and hypertrophy through the signaling pathways mediated by Akt, $p27^{Kip1}$ and p38 MAPK. Because the formation of PIP complex is closely associated with regulation of the mesangial cell behaviors including cell adhesion and proliferation, the present study suggests a novel regulatory mechanism, in which TGF-β1 can influence the proliferation and hypertrophy of GMCs through modifying the PIP complex and thereby contributes to the development and/or progression of glomerular failure.

Although several lines of evidence suggest the important role of TGF-β1, which level is often increased in injured glomeruli, in the progression of glomerular diseases, the cellular and molecular mechanisms underlying the proliferative and hypertrophic effects of TGF-β1 are not fully understood. Because evaluating the molecular mechanisms involved in the assembly and function of the PIP complex are considerable value for understanding cellular pathogenesis of glomerular injury, we were particularly interested in the role of the PIP complex in mediating the proliferative and hypertrophic effects of TGF-β1. Guo and Wu (2002) demonstrated the reduction in fibronectin depo-

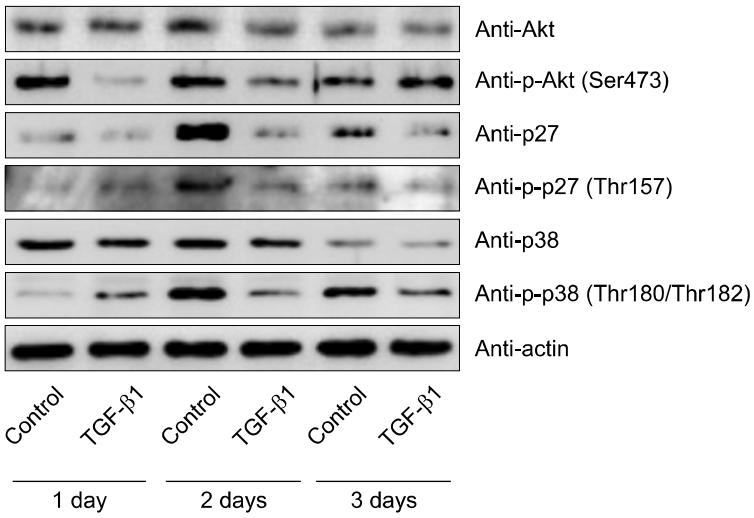

Figure 5. Alteration of TGF-β1-induced phosphorylation of Akt, $p27^{Kip1}$ and p38 MAPK in cultured rat glomerular mesangial cells. Cells were cultured under 1% serum-contained condition with or without TGF-β1 (2 ng/ml) for 1-3 days. Cell lysates were prepared as described in the Materials and Methods and analyzed by Western blotting with antibodies recognizing phospho-Akt (Ser 473), phospho-$p27^{Kip1}$ (Thr 157) phospho-p38 MAPK (Thr 180/Thr 182) and actin. Note that a decreased Akt phosphorylation and an increased phosphorylation of $p27^{Kip1}$ and p38 MAPK are observed at day 1 after treatment of TGF-β1, whereas these cellular responses to TGF-β1 are completely reversed on day 3.

sition and proliferation of GMCs by inhibiting the PIP complex formation, suggesting that the PIP complex likely represents a key regulator of the mesangial cell behavior. Our previous report (Jung *et al*., 2007) demonstrated that TGF-β1 differently regulates the PIP complex formation in podocytes and GMCs, and the TGF-β1-induced up- and down-regulation of the PIP complex likely contribute to the pleiotropic effects of TGF-β1 on different glomerular cell types and hence the progression of glomerular failure. This study observed the TGF-β1-induced biphasic change of the PIP complex formation in GMCs, showing that TGF-β1 causes early increase of the PIP complex formation with subsequent reduction (Figure 1A). Taken together with the present results and previous reports, it is considerable that TGF-β1 not only regulates the PIP complex formation in cell type-specific fashion, but also modulates in time-dependent manner. Additionally, because the PIP complex plays a crucial role in the regulation of mesangial cell behavior (Guo and Wu, 2002), and TGF-β1 causes early and self-limited proliferation of GMCs with subsequent hypertrophy (Wolf and Ziyadeh, 1999), we also consider that biphasic alteration of the PIP complex formation by TGF-β1 may be implicated, at least in part, in the proliferation and hypertrophy of GMCs.

Although several studies suggested the pathological importance of mesangial cell proliferation by TGF-β1 in the early stage of diabetic glomerulopathy (Wolf and Ziyadeh, 1999; Bak *et al*., 2000), the effect of TGF-β1 on mesangial cell proliferation is still controversial. The previous studies have shown that TGF-β1 results in transient proliferation of rats GMCs, followed by growth inhibition (Park *et al*., 2000; Drummond and Mauer, 2002), and similar results were also observed in our previous study (Rho *et al*., 2004). In contrast, TGF-β1 inhibits the proliferation of human and mouse GMCs which were cultured under serum-free condition (Stephenson *et al*., 1998). These reports indicate that TGF-β1 regulates mesangial cell proliferation in a species-specific and experimental condition-dependent fashion. Because many cells cultured under serum-deprived condition possibly undergo apoptotic cell death (Jekabsons and Nicholls, 2006; Yun *et al*., 2006), GMCs in this study were treated with TGF-β1 for 1-3 days under 1% serum-contained condition. A significant increase in cell proliferation and hypertrophy was observed at 2 and 3 days after treatment of TGF-β1, respectively. These results demonstrate that TGF-β1 induces early and self-limited proliferation of GMCs with subsequent hypertrophy, which is characterized as a pathological process of GMCs observed in the many glomerular diseases. Additionally, we consider that TGF-β1-induced alteration of the PIP complex formation likely plays an important role in regulation of mesangial cell proliferation and hypertrophy based on the following evidences: First, TGF-β1-induced up- and down-regulation of the PIP complex formation were occurred at 1 and 2 days after TGF-β1 treatment, respectively (Figure 1B), and a significant increase in mesangial cell proliferation and hypertrophy was observed at 2 and 3 days after TGF-β1 treatment (Figure 2). Second, several lines of evidence suggest that the PIP complex likely represents a key regulator of the glomerular cell behavior (Guo and Wu, 2002; Jung *et al*., 2007). Thus, TGF-β1-PIP complex pathway possibly functions as an upstream regulator of mesangial cell proliferation and

hypertrophy by TGF-β1.

TGF-β1 level significantly increases in the kidney with diabetic nephropathy, and diabetic nephropathy encompasses discrete structural alterations of GMCs including mesangial expansion and hypertrophy. *In vitro* study, upon exposure to high glucose, GMCs enter the cell cycle and exhibit a biphasic growth response (Young *et al*., 1995; Rho *et al*., 2004), suggesting that the initial proliferative phase is followed by G_1 arrest of cell cycle with progressive hypertrophy. One of biochemical mechanisms underlying cellular hypertrophy is cell cycle arrest at the G_1/S checkpoint, leading to increased protein content to cell number ratio (Wolf and Shankland, 2003), which is a biochemical definition of hypertrophy. Interestingly, the results presented in this paper show that mesangial cell proliferation dramatically reduced at 3 days after treatment of TGF-β1, but cell hypertrophy significantly elevated in the same cells. These results can explain the cellular mechanism involved in glomerular enlargement, which occurs virtually in every type of chronic kidney diseases. This suggestion can be also supported by the previous report (Wolf and Ziyadeh, 1999), suggesting that the predicted evolution of diabetic glomerulopathy in both *in vivo* and cell culture study is comprised of an early, self-limited degree of mesangial cell proliferation, followed by the development of hypertrophy of these cells that herald the slow progression into glomerulosclerosis.

Because it has been known that the residual mesangial cells in response to loss of GMCs often undergo compensatory hypertrophy to maintain physiological function (Hostetter, 1995), this study estimated whether TGF-β1 might cause apoptotic cell death. Treatment of GMCs with TGF-β1 significantly increased the caspase-3 activity at early stage (Figure 3), indicating that TGF-β1 acts as an upstream regulator for modulating the caspase-3 activity in cultured rat GMCs. Therefore, we consider that mesangial cell hypertrophy observed in this study is likely associated with the compensatory response of residual cells to apoptotic cell death. Additionally, this study also suggests that the early increase in the PIP complex formation by TGF-β1 is possibly implicated in the regulation of cell proliferation, and TGF-β1-induced early increase of caspase-3 activity, especially on day 1, may be independently mediated by the signaling pathways which are involved in the TGF-β1-PIP complex-cell proliferation pathway, because it has been reported that ILK couples PINCH-1 and α-parvin to downstream signaling pathways involved in the suppression of apoptosis and in promoting cell cycle progression (Dedhar, 2000; Wu, 2004).

Several lines of evidence suggested that $p27^{Kip1}$ is a putative tumor suppressor, and cellular responses including proliferation, apoptosis and hypertrophy to injury are ultimately governed at the nuclear level of cell cycle-regulating proteins such as $p27^{Kip1}$ (Blagosklonny, 2002; Wolf and Shankland, 2003). When GMCs were incubated with TGF-β1 for 1 day, cytoplasmic expression of $p27^{Kip1}$ increased, but its nuclear expression significantly decreased. Interestingly, these expressional patterns of $p27^{Kip1}$ in cytoplasmic and nuclear fractions were completely reversed in GMCs treated with TGF-β1 for 2 days. Thus, it is considerable that intracellular localization of $p27^{Kip1}$ may critically regulate the proliferation and hypertrophy of the cultured rat GMCs. Additionally, total level of $p27^{Kip1}$ expression increased at 1 day after treatment of TGF-β1, but decreased at the other day tested in this study, suggesting that $p27^{Kip1}$ likely functions to protects the GMCs from apoptosis, because when $p27^{Kip1}$ levels are maintained in glomerular diseases such as membranoproliferative glomerulopathy and diabetic nephropathy (Shankland *et al*., 1997). Based on these, we suppose that total amount and/or intracellular localization of $p27^{Kip1}$ may be possibly implicated, at least in part, in the TGF-β1-PIP complex pathway which crucially regulates the mesangial cell behavior including proliferation and hypertrophy.

This study further examined whether phosphorylation of cell survival signaling proteins, Akt, $p27^{Kip1}$ and p38, regulates the development of mesangial cell proliferation and hypertrophy through TGF-β1-PIP complex pathway. It is known that loss of ILK, PINCH-1 or α-parvin comprises Akt activation in many, but not all cell types (Wu, 2005; Legate *et al*., 2006). TGF-β1 significantly decreased early Akt phosphorylation (Ser473), followed by returning to the normal level (Figure 5). These results lead us to consider that Akt is not downstream regulator of the TGF-β1-PIP complex pathway as observed in podocytes (Jung *et al*., 2007). This explanation can be also supported by the previous reports, suggesting that involvement of the PIP complex in Akt activation appears to be particularly prominent in cancer cells (Jung *et al*., 2007), and PI3-PDK1-Akt pathway alone is not sufficient to stimulate TGF-β1-induced collagen type I gene expression in GMCs (Runyan *et al*., 2004; Legate *et al*., 2006).

In contrast to TGF-β1-induced intracellular localization of $p27^{Kip1}$ shown in Figure 4, TGF-β1 causes early increase of $p27^{Kip1}$ phosphorylation (Thr 157) with subsequent decrease (Figure 5). It

has been reported that phosphorylation of $p27^{Kip1}$ causes retention of $p27^{Kip1}$ in the cytoplasm, thereby inactivating its ability to inhibit CDK2 in the nucleus (Blagosklonny, 2002; Fujita *et al*., 2002). Based on these, up- and down-regulation of $p27^{Kip1}$ phosphorylation by TGF-β1 may be closely associated with the intracellular translocation of $p27^{Kip1}$ as shown in Figure 4. Thus, it is considerable that TGF-β1-PIP complex pathway regulates the $p27^{Kip1}$ phosphorylation, and nuclear localization of $p27^{Kip1}$ is negatively correlated with $p27^{Kip1}$ phosphorylation in GMCs treated with TGF-β1. Therefore, this study suggests that TGF-β1-PIP complex-$p27^{Kip1}$ pathway is possibly implicated in the regulation of mesangial cell proliferation and hypertrophy.

Furthermore, this study tested involvement of p38 MAPK, which is one of crucial cell survival intermediates, in the regulation of mesangial cell proliferation and hypertrophy through TGF-β1-PIP complex pathway. We previously reported that TGF-β1-induced disruption of the PIP complex increased the p38 MAPK phosphorylation (Thr 180/Thr 182) in podocytes (Jung *et al*., 2007). Interestingly, this study observed a biphasic change of p38 MAPK phosphorylation in GMCs, showing that TGF-β1 caused early increase of p38 MAPK phosphorylation with subsequent decrease (Figure 5). Taken together, these results suggest that TGF-β1-induced alteration of the PIP complex formation positively modulates the phosphorylation of p38 MAPK, and this TGF-β1-PIP complex-p38 MAPK pathway likely contributes to the development of mesangial cell proliferation and hypertrophy. Additionally, we also suggest that the PIP complex differently modulates the phosphorylation of p38 MAPK in cell type-specific fashion, because TGF-β1, under the same condition, promotes the PIP complex formation and p38 MAPK phosphorylation in GMCs (unpublished data), but causes down-regulation of the PIP complex formation and up-regulation of p38 MAPK in podocytes (Jung *et al*., 2007).

In summary, the PIP complex is a downstream regulator of TGF-β1 in cultured rat GMCs, and this TGF-β1-PIP complex pathway regulates mesangial cell proliferation and hypertrophy through mediating cell survival and death signaling intermediates including $p27^{Kip1}$, p38 MAPK and caspase-3. The responses of TGF-β1-induced PIP complex formation and their pathophysiological roles in glomerular cells are cell type specific. Defining these events are valuable for understanding cellular mechanisms involved in the development and progression of glomerular failure and will ultimately lead to novel therapeutic approach that will reduce the incidence of glomerular diseases and other pathological processes involving abnormal cell proliferation and hypertrophy.

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