Expression of α-Actinin-1 in Human Glomerular Mesangial Cells *In Vivo* and *In Vitro*

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Recent studies have demonstrated important roles of α-actinins in glomerular disease, while little information is known about the expression profile of α-actinins in human glomerular mesangial cells. Here, immunofluorescence and confocal microscopy showed that α-actinin-1 exclusively distributed along mesangial cells in human glomeruli of IgA nephropathy. RT-PCR and Western blot further confirmed the expression of α-actinin-1 in primary cultured human mesangial cells. We also found that transforming growth factor-β1 (TGF-β1) stimulated ACTN1 gene transcription and that transiently transfected α-actinin-1 significantly increased TGF-β1-induced plasminogen activator inhibitor-1 (PAI-1) promoter activity in human mesangial cells. These findings suggest that α-actinin-1 may play a role in human glomerular disease. Exp Biol Med 233:689–693, 2008

Key words: α-actinin-1; mesangial cells; transforming growth factor-β1; plasminogen activator inhibitor-1; glomerular disease

Introduction

Glomerular mesangial cells, one of the three major cellular components in renal glomeruli, are considered to play important roles in the pathogenesis of glomerular disease. Exposure to pathological stimuli can initiate the activation of quiescent mesangial cells *in vivo*, which occurs in many human glomerular diseases (1). The activated mesangial cells enhance the production of extracellular matrix (ECM) proteins such as collagen, fibronectin, and plasminogen activator inhibitor-1 (PAI-1), which results in accumulated ECM deposit in glomerular mesangium and irreversible glomerular injury in the end (2, 3).

α-Actinin, an actin-crosslinking protein, is composed of four isoforms in human. The α-actinin-2 and -3 are mainly expressed in the sarcomere (4), while the non-muscle isoforms, α-actinin-1 and -4, are widely distributed (5, 6). Increasing attention has been focused on the role of non-muscle α-actinins in glomerular disease, since α-actinin-4 site mutations was reported to cause familial focal and segmental glomerulosclerosis in 2000 (7). α-Actinin-1 has been detected in primary cultured podocytes (7–9), while there is no report yet whether α-actinin-1 is expressed in human mesangial cells.

In our present study, the exclusive distribution of α-actinin-1 in mesangial cells was detected in human biopsies of patients with IgA nephropathy, focal segmental glomerulosclerosis, minimal change disease, and thin glomerular basement membranes. α-Actinin-1 expression was also detected in primary cultured human mesangial cells (HMC). We have also found that transforming growth factor-β1 (TGF-β1) stimulated ACTN1 gene transcription and that transiently transfected α-actinin-1 significantly increased TGF-β1-induced PAI-1 promoter activities. These findings suggest that α-actinin-1 may play a role in human glomerular disease.

Materials and Methods

Materials. Human TGF-β1 was purchased from R&D (Minneapolis, MN, USA). Primary antibodies were purchased from the following vendors: mouse anti-actinin-1 (sc-17829, Santa Cruz Biotechnology, CA, USA); anti-actinin-4 (0042-05, ImmunoGlobe, Germany); mouse anti-α-SMA FITC-labeled (F3777, Sigma, MO, USA). Alexa Fluor labeled secondary antibodies were from Invitrogen (Carlsbad, CA, USA).

The study was supported by grants to W. F. G. from the National Kidney Foundation of the Virginias (NKFVA019) and the Norman S. Coplon Extramural Grant from Satellite Research.

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Received October 16, 2007.
Accepted January 18, 2008.

DOI: 10.3181/0710-RM-279
1535-3702/08/2336-0689$15.00

Table 1. RT-PCR Primers and Products

Gene	Direction	Primer sequence (5′... 3′)	Product (bp)
ACTN1	Forward	TCATCTCAGGTGAACGCTTG	207
	Reverse	AGATGTCCTGGATGGCAAAG	
ACTN2	Forward	CCTTCGCTTTGCTATTCAGG	212
	Reverse	TGGGGTCATCCTTGTTAAGC	
ACTN3	Forward	AATCGCCAACGTTAACAAGG	355
	Reverse	AGTGTTCAGGTTTCCGATGG	
ACTN4	Forward	AGTATGAACGCAGCATCGTG	194
	Reverse	GGTGAGGATCTGGTTCTCCA	
GAPDH	Forward	GGTGAAGGTCGGAGTCAACG	500
	Reverse	CAAAGTTGTCATGGATGACC	

Cell Culture and Transient Transfection. HMC were isolated and cultured as previously described (10). Luciferase reporter gene driven by human PAI-1 promoter (pPAI-1-Luc) was constructed as shown before (11). HMC were transiently transfected with pPAI-1-Luc vector and human recombinant α-actinin-1 plasmid using Fugene 6 reagent (Roche). Three hours after transfection, the cells were stimulated with 10ng/ml TGF-β1 for an additional 24 h. Cells were then lysed and luciferase activity was read using TD20/20 luminometer (Turner Diagnostics). In all transfection experiments, phRL-TK was co-transfected as an internal control for normalization of transfection efficiency.

Human Biopsies of Glomerular Disease and Immunofluorescence. Human biopsies were obtained from patients with glomerular disease (Department of Pathology, Eastern Virginia Medical School, Virginia, USA) under an IRB-approved protocol. Biopsies diagnosis was based on regular microscopic assay, electronic microscopy, and immunofluorescence. For immunofluorescence assay, all sections were blocked for 1 h at room temperature. Subsequently, primary antibodies were applied for 1 h at room temperature. After washed with PBS, the sections were then incubated with Alexa Fluor labeled secondary antibodies at room temperature for 1 h. For actinin-1 and α-SMA double staining, mouse anti-α-SMA FITC-labeled antibody was applied after actinin-1 staining. Slides were then mounted with Fluoromount-G (SouthernBiotech). Fluorescent images were captured with confocal laser scanning microscopy (Zeiss 510).

RNA Isolation and RT-PCR. HMC total cellular RNA was isolated using TRIzol (Invitrogen). Amplification of transcripts were performed using 100 ng of total RNA and one-step reverse transcription RT-PCR system (Qiagen) according to the manufacturing protocol. The PCR primer sequences used in this study were designed using Primer3 software and are presented in Table 1.

Immunoblotting. HMC were lysed in RIPA buffer containing protease inhibitors. Five μg protein/lane was resolved on a 10% SDS-PAGE gel. After transferred onto PVDF membranes and blocked, the membranes were incubated with the indicated diluted primary antibody overnight at 4°C. Membranes were then incubated with appropriate horseradish peroxidase-conjugated secondary antibody for 1h at room temperature. Immunoreactive bands were detected by ECL reagents (Amersham Bioscience) and exposed to X-ray film.

Statistical Analysis. Data are presented as mean ± SEM, and represent the averages of at least three independent experiments. Differences between the mean values were analyzed by Student's t test. A P value of <0.05 was considered significant.

Results

The Distribution of α-Actinin-1 in Mesangial Cells Was Detected in Human Glomerular Disease. Immunofluorescence staining and confocal microscopy were used to examine the localization of α-actinin-1, α-actinin-4 and α-SMA in biopsies of patients with IgA nephropathy (n = 4), focal segmental glomerulosclerosis (n = 4), minimal change disease (n = 8) and thin glomerular basement membranes (n = 4). More than 75% glomeruli showed α-actinin-1 staining. Possible due to insufficient numbers of cases, statistically significant differences in the intensity of α-actinin-1 staining were not observed, but α-actinin-1 was consistently mesangial and colocalized with α-SMA whenever it was present. Figure 1 demonstrates that α-actinin-1 was expressed and exclusively localized in mesangial cells in an example of a biopsy of IgA nephropathy. The distribution of α-actinin-1 in mesangial cells was confirmed by the colocalization of α-actinin-1 with α-SMA, a marker of mesangial cells activation (Fig. 1D, E, F). We found that α-actinin-4 mainly localized to podocytes (Fig. 1A, B, C), as there is no colocalization of α-actinin-4 with α-SMA in glomeruli (Fig. 1G, H, I).

α-Actinin-1 Was Expressed and Induced by TGF-β1 in Primary Cultured HMC. Basal α-actinin-1 expression in primary cultured HMC was detected by RT-PCR and Western blot (Fig. 2A, B). α-Actinin-4 expression in mesangial cells was also detected at mRNA level, while no expression of α-actinin-2, 3 was found (Fig. 2A). We also found that TGF-β1 significantly increased ACTN1 gene transcription as early as 4 h (Fig. 2C).

α-Actinin-1 Promoted TGF-β1-Induced PAI-1 Promoter Activity in HMC. PAI-1, a major component of ECM accumulated in sclerotic glomeruli, has been

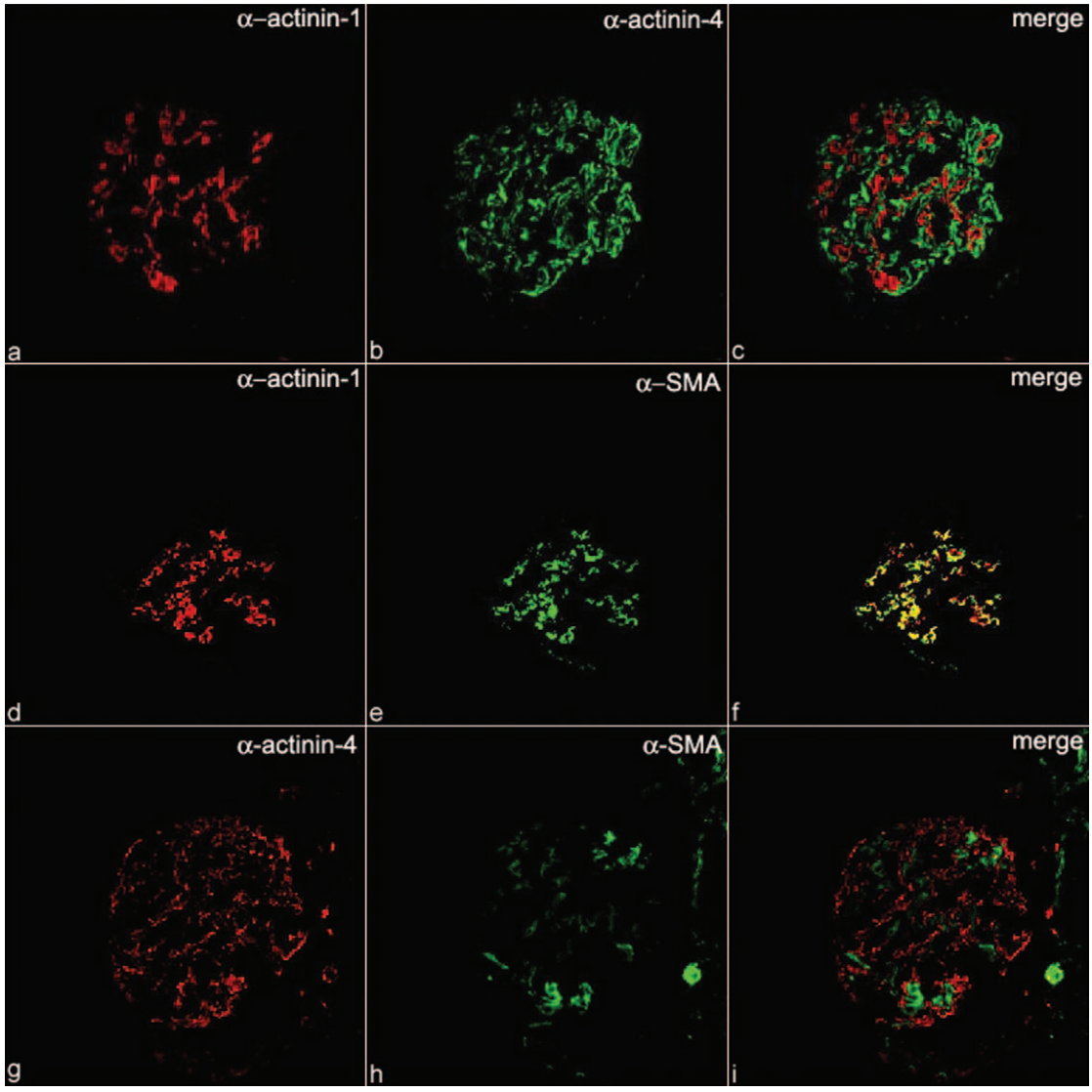

Figure 1. The distribution of α-actinin-1 in mesangial cells was detected in human glomerular disease. Distribution of α-actinin-1, α-actinin-4 and α-SMA in glomeruli of IgA nephropathy was examined by immunofluorescence staining and confocal microscopy. A color version of this figure is available in the online journal.

reported to play important roles in the pathogenesis of glomerular disease (12). TGF-β1 is a main inducer of PAI-1 expression. We therefore examined whether α-actinin-1 participated in TGF-β1-mediated PAI-1 gene expression. We cloned human PAI-1 promoter region into a luciferase reporter gene containing vector. As shown in Figure 3, TGF-β1 stimulated PAI-1 promoter activity ($P < 0.001$) and transiently transfected α-actinin-1 increased TGF-β1-induced PAI-1 promoter activity by 6.3 fold ($P < 0.001$).

Discussion

Previous investigations have shown that α-actinin-4 participates in the development of some glomerular diseases (13, 14), while little information is available about the role of the other non-muscle α-actinin, α-actinin-1, in human glomerular disease. We are the first to demonstrate that α-actinin-1 is expressed in glomerular mesangium in IgA nephropathy as well as other conditions. The exclusive distribution of α-actinin-1 in mesangial cells was further confirmed by the colocalization of α-actinin-1 with α-SMA. α-SMA expression can only be detected in activated mesangial cells, which occurred in glomerular disease and primary cultured mesangial cells *in vitro* (15, 16). No α-SMA is expressed in normal adult mesangial cells *in situ* (17). α-Actinin-1 has been reported as minimally detectable in normal kidney (7). In our study, α-actinin-1 was detectable in the mesangium of all biopsies except for a single biopsy from a patient with minimal change disease. This suggests that α-actinin-1 may be a more sensitive indicator than α-SMA of mild mesangial cell activation or that our staining methodology is more sensitive than the published studies. Further investigation with increased

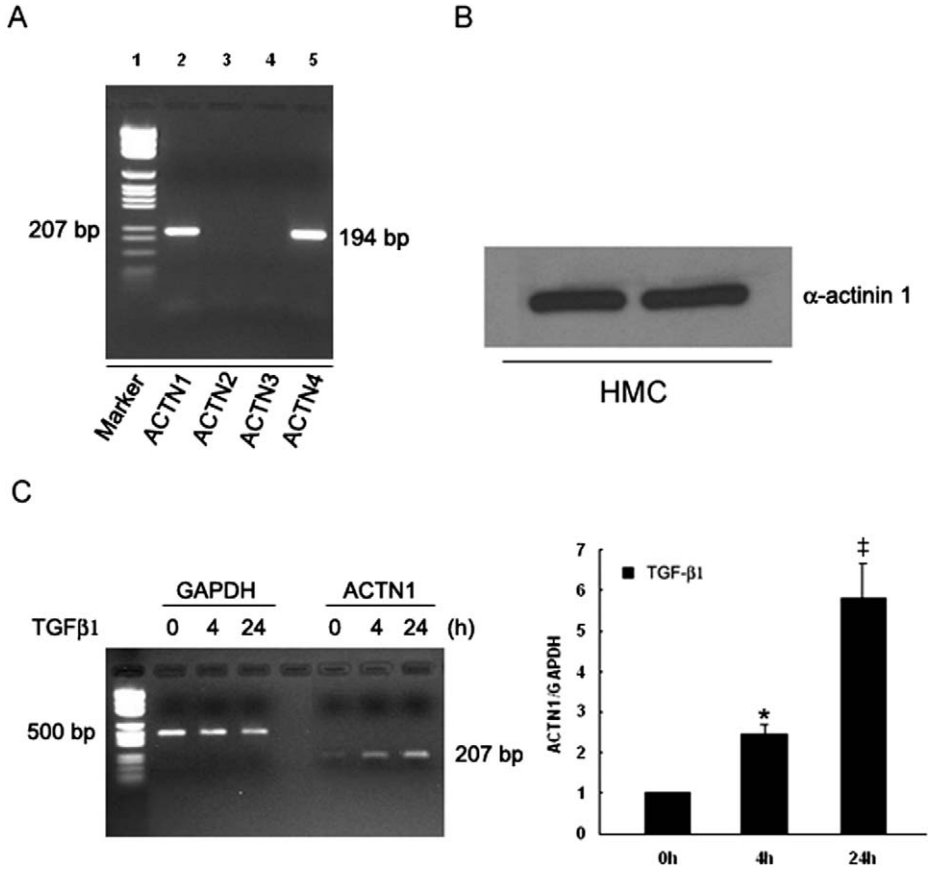

Figure 2. α-Actinin-1 was expressed and induced by TGF-β1 in primary cultured human mesangial cells. A: RT-PCR yielded a 207-bp product for α-actinin-1 (lane1) and a 194-bp product for α-actinin-4 (lane 4). No α-actinin-2 and α-actinin-3 expression was detected (lane 2, 3). B: Western blot with specific antibody also confirmed α-actinin-1 expression in two different sets of mesangial cells. C: Serum-starved HMC were treated with TGF-β1 for 0, 4, and 24 h. RT-PCR was performed to measure the changes of α-actinin-1 mRNA level. Representative gel profile was given (left). Summarized results for the time course of TGF-β1-induced α-actinin-1 mRNA transcription was presented (right). *, $P < 0.01$ versus 0 h; ‡, $P < 0.001$ versus 0 h.

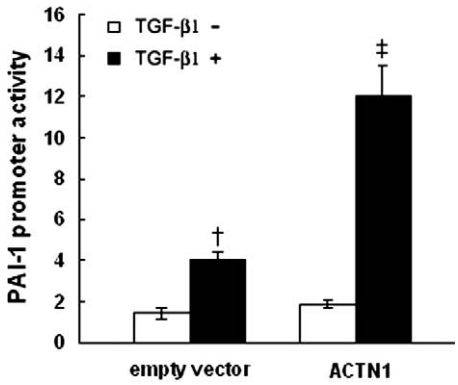

Figure 3. α-Actinin-1 promoted TGF-β1-induced PAI-1 promoter activity. HMC were co-transfected with pPAI-1-Luc, α-actinin-1 plasmid or empty vector, together with phRL-TK as an internal control. Three hours after transfection, the cells were further stimulated with 10 ng/ml TGF-β1 for another 24 h. Promoter activity was read, as described in Materials and Methods. †, $P < 0.001$ versus untreated control; ‡, $P < 0.001$ versus TGF-β1 treated control.

numbers of specimens, including tissue from "normal" nephrectomy specimens may clarify this issue.

Zhao *et al.* (18) demonstrated that primary mouse mesangial cells derived from the lupus prone MRL-lpr/lpr strain have higher expression levels of α-actinin-1 and -4, suggesting that enhanced non-muscle α-actinin expression may be important in the pathogenesis of lupus nephritis. The facts that α-actinin-4 was expressed in primary cultured mesangial cell, while the localization of α-actinin-4 *in vivo* was almost restricted to podocytes show that α-actinin-1 is the dominant non-muscle isotype in mesangial cells *in vivo*. α-Actinin-1, rather than α-actinin-4, may play more important roles in glomerular diseases associated with mesangial injury.

TGF-β1 has been considered to be a critical mediator in mesangial activation and the progression of glomerulosclerosis (19). Our previous studies have showed that TGF-β1 stimulated α-SMA expression in mesangial cells (16). Here, we found that TGF-β1 also stimulated α-actinin-1 gene expression in mesangial cells. This supports the possibility that α-actinin-1 may be a sensitive marker of mesangial injury and activation.

PAI-1, not expressed in normal kidney, is highly induced in many glomerular diseases (20, 21). Previously, we showed that TGF-β1 can increase PAI-1 expression in mesangial cells (11). Here, we found not only TGF-β1 increased α-actinin-1 but also overexpression of α-actinin-1 stimulated TGF-β1-induced PAI-1 promoter activity in mesangial cells. Besides binding actin filaments, α-actinin-1 is also a multifunctional protein. α-Actinin has been reported to interact directly with more than 20 cellular proteins of great functional diversity, among which many are important signal molecules (22). α-Actinin was found to interact with ERK (23). Both Smads and MAPKs pathways have been demonstrated participating in TGF-β1-stimulated PAI-1 expression (24, 25). Therefore, α-actinin-1 may regulate PAI-1 gene expression by modulating the activation of TGF-β1 downstream signal molecules such as Smads and or MAPKs, thus accelerating ECM accumulation in glomerular disease.

Our study suggests that α-actinin-1 plays a role in the activation of mesangial cells. α-Actinin-1 may be a sensitive marker of mesangial cell injury to be used in the diagnosis and prognosis of human glomerular disease.

We thank Dr. Beatrice Haimovich (Department of Surgery, University of Medicine and Dentistry of New Jersey) for providing human recombinant α-actinin-1 plasmid.

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