Short Communication

Calponin Expression in Renal Tubulointerstitial Fibrosis Induced in Rats by Cisplatin

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Abstract: Renal tubulointerstitial fibrosis is the common feature of chronic renal failure, regardless of its etiology. Myofibroblasts play important roles in progression of the fibrosis and are characterized by expressions of various cytoskeletons such as vimentin, desmin and α-smooth muscle actin (α-SMA). To pursue the characteristics of the cells, we immunohistochemically investigated the relationship between calponin (a marker of terminal smooth muscles) expression and myofibroblasts in cisplatin-induced rat renal tubulointerstitial fibrosis. Calponin-expressing interstitial cells increased with fibrosis and reacted simultaneously to vimentin or α-SMA (a marker of well-differentiated myofibroblasts) but not desmin or Thy-1 (a marker of myofibroblasts at the early stage). The present study shows that calponin may be expressed transiently in relatively well-developed myofibroblasts in rat renal fibrosis. Calponin could become a marker for myofibroblast development in chronic renal toxicity in rats. (DOI: 10.1293/tox.2013-0048; J Toxicol Pathol 2014; 27: 97–103)

Key words: calponin, myofibroblasts, tubulointerstitial fibrosis, cisplatin, rat

Regardless of the underlying causes, renal tubulointerstitial fibrosis is the common feature of chronic renal failure1. The lesion is characterized by excessive accumulation of extracellular matrices (ECMs), such as collagens and fibronectin, which may be produced by renal interstitial mesenchymal cells. Of these cells, myofibroblasts play important roles in progression of the fibrosis, because the cells are regarded as the major source of ECMs2. Myofibroblasts are characterized by expressions of various cytoskeletons such as vimentin, desmin and α-smooth muscle actin (α-SMA)3, 4. They have been postulated to originate from resident fibroblasts and bone-marrow-derived precursors or to be formed through epithelial mesenchymal transition (EMT) in which injured renal epithelial cells acquire mesenchymal myofibroblastic phenotypes5–8. Despite the important role of myofibroblasts in renal fibrosis, their precise origin and nature remain to be investigated.

Calponin is a marker of terminal smooth muscle differentiation, and its expression is restricted to smooth muscle cells; the protein is thought to be involved in the regulation of smooth muscle contraction9, 10. Recently, calponin was found to be expressed in myofibroblasts in human pulmonary fibrosis11 and in periglomerular myofibroblasts in rat glomerulopathy12. However, the expression of calponin in myofibroblasts appearing in tubulointerstitial fibrosis has yet to be investigated.

Previously, we showed that myofibroblasts in cisplatin (CDDP)-induced rat renal interstitial fibrosis could exhibit varying degrees of expression of vimentin, desmin and α-SMA13. To shed some light on the pathogenesis of renal tubulointerstitial fibrosis, in this study, we investigated calponin expression in correlation with vimentin, desmin or α-SMA expressions by using immunolabeling. In addition, we carried out comparisons of calponin and Thy-1 expressions, because Thy-1, a GPI-anchored cell surface protein, is regarded as a possible marker of early myofibroblast development13.

Forty-two 6-week-old male F344/DuCrj rats (Charles River Laboratories Japan, Hino, Shiga, Japan) were injected intraperitoneally with a single dose (6 mg/kg body weight) of CDDP (Nippon Kayaku Co. Ltd., Tokyo, Japan)13. They were housed in an animal room controlled at 22 ± 3°C with a 12 hour light-dark cycle and allowed free access to a standard commercial diet (MF, Oriental Yeast Co. Ltd., Tokyo) and tap water. They were euthanized by exsanguination under anesthesia, and kidney samples of three rats were obtained on days 1, 3, 5, 7, 9, 12, 15, 20, 25, 35, 45 and 60 after CDDP injection. Control rats were injected with an equivalent volume of phosphate buffered saline (PBS), and three rats were sacrificed on day 0 (on injection day) and on day 60. All the experimental protocols and animal housing conformed to the institutional guidelines of Osaka Prefecture University for animal care.

For the immunohistochemical analyses, renal samples...
were fixed in periodate-lysine-paraformaldehyde (PLP) fixative, embedded in paraffin by the AMeX method (PLP-AMeX method) and sectioned at 3–4 μm thick. The peroxidase-labeled amino acid polymer method was used with the following primary monoclonal antibodies: anti-calponin monoclonal antibody (Thermo Scientific, Rockford, IL, USA; 1 in 200 dilution), anti-α-SMA monoclonal antibody (Dako, Carpinteria, CA, USA; 1 in 200), anti-vimentin monoclonal antibody (Dako; 1 in 200) and anti-desmin monoclonal antibody (Dako; 1 in 200). For antigen retrieval, deparaffinized sections for vimentin and desmin were pretreated in 10 mM citrate buffer (pH 6.0) with a microwave for 10 min, and sections for calponin and α-SMA were incubated with 0.1% trypsin in PBS (pH 7.4, 0.1 M) for 10 min at 37°C. After pretreatment, sections were incubated with 3% H₂O₂ for 10 min to quench endogenous peroxidase and then with 5% skimmed milk in PBS for 30 min; they were then incubated with each primary antibody overnight at 4°C. Next, incubation with a horseradish peroxidase-conjugated polymer (Histofine Simple Stain MAX-PO; Nichirei, Tokyo, Japan) as the secondary antibody was performed for 1 hour. Positive reactions were visualized with 3, 3’-diaminobenzidine tetrahydrochloride (DAB; Vector Laboratories, Burlingame, CA, USA). Non-immunized mouse serum in place of the primary antibody served as the negative control. Sections were counterstained lightly with hematoxylin. The intensity of immunopositive reactions in the injured corticomedullary area was assessed semiquantitatively with scoring criteria (number and intensity) as follows: –, negative; ±, very faintly positive in a few cells; +, faintly positive in some cells; 2+, weakly positive in a moderate number of cells; 3+, moderately positive in a number of cells; 4+, strongly positive in a large number of cells; 5+, very strongly positive in a large number of cells.

For double immunofluorescence analyses, renal samples were embedded in optimum cutting temperature compound and frozen at –80°C for cryosectioning. Frozen sections (7 μm thick) were cut, air-dried and fixed in 1:1 acetone and methanol mixture at –20°C for 5 min. For comparison between calponin and cytoskeletons, after being blocked with normal goat serum, the sections were incubated with calponin polyclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA) and each of the primary monoclonal antibodies against α-SMA, vimentin or desmin for 1 hour. After washing in PBS, sections were reacted with goat anti-rabbit IgG secondary antibody conjugated to Alexa Flour® 568 (Invitrogen; 1 in 1,000) for calponin or the goat anti-mouse IgG secondary antibody conjugated to Alexa Fluor 488 (Invitrogen, Carlsbad, CA, USA; 1 in 1,000) for α-SMA, vimentin, desmin and Thy-1 (Cedarlane Laboratories Ltd., Burlington, ON, Canada; 1 in 100 dilution) for 1 hour. All slides were mounted with mounting medium containing 4’,6-diamino-2-phenylindole (DAPI; VECTASHIELD; Vector Laboratories, Burlingame, CA, USA) for nuclei. Signals were detected and analyzed with a confocal laser scanning microscope (C1Si; Nikon, Tokyo, Japan). Negative controls for the immunolabelings were confirmed either by omission of the primary antibody or by treatment with a non-immunized mouse and rabbit IgG.

CDDP-induced renal interstitial fibrosis following renal tubular injury has been previously described. Briefly, after cell injury, necrosis and desquamation on post-CDDP injection days 1, 3 and 5, renal proximal tubules, especially the S3 segment in the cortico-medullary junctions, underwent regeneration with cell infiltration around the affected tubules on day 7; the regenerating epithelial cells were cuboidal or flattened in shape and had basophilic cytoplasm. On days 9–35, interstitial fibrosis began to be seen around the affected tubules, consisting of spindle-shaped fibroblastic cells; the degree gradually progressed with time, with the greatest degree observed on day 35. On days 45 and 60, cellular elements in the fibrotic lesions showed gradual decreases, resulting in scar formation.

In control kidneys, vascular smooth muscle cells gave a strong positive reaction to calponin; however, there were no interstitial cells reacting to calponin in the cortex and
the medulla (Fig. 1). On days 3–7, calponin was faintly expressed in a few cells in the interstitium of the affected cortico-medullary junction (Fig. 2a). On day 9, calponin-positive cells began to be clearly seen around the affected renal tubules with abnormally dilated lumina and interstitial fibrosis. More interestingly, some regenerating cuboidal or flattened epithelial cells lining the abnormally dilated tubules showed a positive reaction to calponin (Figs. 2b and 2c), although normal epithelial cells did not react to calponin. On days 12–20, the calponin-positive interstitial cell number was markedly increased, showing the highest level (Table 1); in the extremely dilated renal tubules, spindle-
Table 1. Immunohistochemical Reactivity to Cytoskeletal Markers, Including Vimentin, Desmin and α-Smooth Muscle Actin (α-SMA), and Calponin in the Cortico-medullary Junction in CDDP-induced Rat Renal Interstitial Fibrosis

| Antibody to         | Control | 1   | 3   | 5   | 7   | 9   | 12  | 15  | 20  | 25  | 35  | 45  | 60  |
|---------------------|---------|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|-----|
| Vimentin*           | –       | ±   | ±   | 3+  | 3+  | 3+  | 3+  | 4+  | 4+  | 5+  | 5+  | 5+  | 3+  |
| Desmin*             | –       | ±   | ±   | ±   | 2+  | 3+  | 3+  | 3+  | 3+  | 3+  | 2+  | 2+  | 2+  |
| α-SMA*              | –       | –   | –   | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   | ±   |
| Calponin            | –       | –   | –   | ±   | ±   | ±   | ±   | 2+  | 3+  | 3+  | 2+  | 2+  | 2+  |

Scoring criteria (see text). *These data were cited from our previous article13.

Myofibroblasts play an important role in progression of fibrosis by excessively producing ECMs15. It is well known that myofibroblasts can heterogeneously express cytoskeletal markers such as vimentin, desmin and α-SMA in fibrosis depending on the progression and location of the lesions15–17. Therefore, myofibroblasts are divided into following types: cells expressing only vimentin (V type), those expressing vimentin and desmin (VD type), those expressing vimentin and α-SMA (VA type) and those expressing vimentin, α-SMA and desmin (VAD types). α-SMA is a canonical marker for well-developed myofibroblasts in wound healing1, 16, 17. By single and double immunolabelings, we previously showed that myofibroblasts variously exhibited these cytoskeletons in CDDP-induced rat renal interstitial fibrosis; there were renal myofibroblasts reacting to both vimentin and desmin, desmin and α-SMA or α-SMA and vimentin13.

Myofibroblasts partly have the nature of smooth muscle cells, as they are called contractile cells. Calponin is a marker of smooth muscle differentiation and regulates smooth muscle cell contraction6, 10. In the present study, we showed that myofibroblasts in the progressive renal interstitial fibrosis reacted to calponin; there is also a previous report on calponin expression in periglomerular myofibroblasts in rat glomerular lesions12. By double immunofluorescence, it was further found that calponin-positive interstitial cells reacted to vimentin or α-SMA, but not to desmin (Fig. 3).

In control kidneys, pericytes and some interstitial cells in the cortex showed a positive reaction to Thy-1; however, the majority of cortical interstitial cells showed a negative reaction to Thy-1 (Fig. 4a). On the other hand, many interstitial cells in the medulla showed a positive reaction to Thy-1 (Fig. 4b). The distribution and appearance of Thy-1-positive cells in CDDP-induced renal interstitial fibrosis have been described in our previous article13. The Thy-1-positive interstitial cells were gradually increased in the fibrotic lesions with development of interstitial fibrosis in the affected cortico-medullary junction; the Thy-1-positive cells reacted simultaneously to vimentin or desmin, but there were no cells reacting to both Thy-1 and α-SMA; regenerating renal epithelial cells did not show a positive reaction to Thy-1.

Calponin-positive reactions in vascular smooth muscle cells were also confirmed by immunofluorescence (Fig. 4a). In the fibrotic lesions, there were no calponin-positive cells reacting simultaneously to Thy-1, although interstitial cells and regenerating epithelial cells reacted to calponin (Fig. 4c), which is similar to the findings of the DAB immunohistochemistry (Fig. 2).
Fig. 3. Double immunofluorescence for calponin and vimentin, desmin or α-smooth muscle actin (α-SMA) in the affected cortico-medullary junction on days 15. Calponin-positive cells react to vimentin (a) and α-SMA (c), but there are no cells reacting both to calponin and desmin (b). Bar = 50 μm. Green indicates calponin-positive cells (a, b, and c), and red indicates vimentin (a), desmin (b) and α-SMA (c). DAPI for nuclei (blue). Double-positive cells are shown in yellow (arrowheads).

Fig. 4. Double immunofluorescence for calponin and Thy-1 in the cortico-medullary junction in the control or on day 15. In the control kidney, pericytes (large arrows) and some interstitial cells (arrowhead) show a positive reaction to Thy-1 in the cortex (a), whereas many interstitial cells in the medulla react to Thy-1 (b). In addition to vascular smooth muscles (a, small arrow), calponin expression is seen in regenerating epithelial cells of the dilated tubules (c, large arrows) and interstitial myofibroblasts (c, small arrows); there are no cells reacting simultaneously to calponin and Thy-1 (c). Bar = 50 μm. Red indicates calponin-positive cells (a, c), and green indicates Thy-1 reactivity (a, b, c). DAPI for nuclei (blue).
that Thy-1-expressing myofibroblasts did not correspond to calponin-expressing myofibroblasts. Because α-SMA-positive myofibroblasts expressed calponin, calponin might be expressed in relatively well-differentiated myofibroblasts in renal fibrosis of rats. However, it was noted that none of the α-SMA-positive myofibroblasts expressed calponin. In human prostatic hyperplasia characterized by development of interstitial myofibroblasts, the expression of calponin differed from that of α-SMA in the reactive stroma26. Calponin might have been expressed transiently in well-developed myofibroblasts.

It is interesting to note that calponin was expressed in some regenerating epithelial cells of the affected renal tubule, apparently in the dilated renal tubules. The regenerating renal epithelial cells in kidney lesions may undergo the EMT. Myofibroblasts could develop via the EMT. Transforming growth factor-β1 (TGF-β1), the major fibrogenic factor, regulates the EMT by upregulating α-SMA and calponin27. Calponin expression in regenerating renal epithelial cells might have partly reflected the EMT. However, there were no regenerating epithelial cells expressing α-SMA, although vimentin was expressed in such renal epithelial cells5. The significance of calponin expression in renal epithelial cells remains to be elucidated.

In conclusion, we demonstrated for the first time that there are calponin-expressing interstitial myofibroblasts, which increased with the degree of lesions, in CDDP-induced renal interstitial fibrosis in rats. Calponin may be expressed transiently in renal myofibroblasts at the advanced stages, because the protein was expressed partly in α-SMA-positive, well-developed myofibroblasts but not in Thy-1-positive myofibroblasts at the early stage. Calponin could become a marker for myofibroblast development in chronic renal toxicity in rats.

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