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Invasion of Calponin-positive Glomerular Parietal Epithelial Cells into Glomerular Tuft Is Related to the Development of Glomerulosclerosis

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We previously have reported that periglomerular calponin expression of the glomerulosclerotic glomeruli in the chronic nephropathy. To investigate the role of calponin during glomerulosclerosis, we examined the detailed localization pattern of calponin in chronic nephropathy rat model using serial morphometric analysis. Male Sprague-Dawley rats were used, and chronic nephropathy models were established at 8 and 12 weeks after single intraperitoneal injection of adriamycin (10 mg/kg body weight; n=5). In nephropathy models, 16.3% (8 weeks) and 23.4% (12 weeks) glomeruli showed calponin-positivity at glomerular area. In all these glomeruli, showing various sclerotic changes, calponin-immunoreactivities were present only both the glomerular parietal epithelial cells (PECs) and periglomerular myofibroblasts (PMFs). However, in the glomeruli with weak calponin-positive, immunoreactivity was mostly detected in PECs, suggesting that calponin may be expressed in PECs earlier than in PMFs in the glomerulosclerotic change. Some calponin-positive PECs invaded glomerular tuft with loop-shaped projection, and around this projection, nestin expression of glomerular tuft were much reduced. These results suggested that calponin-positive PECs may play a key role in the development of glomerulosclerosis, and direct contact with PECs and glomerular tuft may be more important to degenerative changes of glomeruli.

Key Words: Glomerulosclerosis, Calponin, Glomerular parietal epithelial cell, Immunohistochemistry, Morphometric analysis

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INTRODUCTION

Most types of chronic renal renal diseases are characterized by the histological and functional end point referred to as end-stage renal disease. Histologically, end-stage renal disease manifests as glomerulosclerosis, vascular sclerosis, and tubulointerstitial fibrosis, with tubulointerstitial fibrosis having consistently been shown to be the most accurate histological predictor of disease progression (Bohle et al., 1987). Among these phenomena, glomerulosclerosis is a unique and endpoint histologic pattern of chronic renal disease regardless of the causes of initial renal damages. Thus it is assumed that some common mechanism(s) may be secondarily concerned with progression of glomerulosclerosis (El Nahas, 1996; Pippin et al., 2009). However, such mechanism(s) are still unclear although lots of studies on the glomerulosclerosis have been done (de Mik et al., 2013). We recently reported that calponin-immunoreactivity was expressed in the periglomerular area of sclerotic glomeruli but not in the non-sclerotic glomeruli using two chronic experimental models, puromycin aminonucleoside nephropathy and 6/5 subtotal nephropathy. In addition, our previous study also showed the thickening of parietal epithelial cells (PECs) covered by calponin-positive periglomerular

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myofibroblasts (PMFs) suggesting interaction between PMFs and PECs of the periglomerular calponin-positive glomeruli (Lee et al., 2010). Therefore, the present study focused on detailed localization pattern of calponin in adriamycin-treated nephropathy rat model using serial morphometric analysis to examine the role of calponin during the glomerulosclerosis, and here we report observations that calponin-positive PECs invaded glomerular tuft with loop-shaped projection, and around this projection, nestin expression of glomerular tuft were much reduced.

MATERIALS AND METHODS

Animals and Tissue Preparation

All experimental procedures conformed to the guidelines of The Catholic University of Kora ethics committee. Young adult male Sprague-Dawley rats (Orient Bio Inc., Korea) of approximate 210~230 g body weight were used, and the animals were cared under a 12 hours light /dark cycle, with standard chow and water ad libitum.

Adriamycin nephropathy (ADN) model was induced by a single intravenous injection to the femoral vein with adriamycin (n=6 per group, doxorubicin hydrochloride; Sigma Chemicals, USA; 10 mg/kg body weight) or sterile saline (sham, n=6; 12 weeks only) under anesthesia. Animals were sacrificed at 8 and 12 weeks after injection after weighing body weight. Under anesthesia, blood was collected via the abdominal aorta. The rats were then briefly perfused with phosphate-buffered saline to remove the blood. The kidney was perfused with 4% paraformaldehyde for 10 minutes, removed, and post-fixed for 3 hours in the same fixative at 4°C. After fixation, The one kidney was processed for wax sections, the other kidney were cut to 50-μm-thick vibratome sections, and processed for preembedding immunohistochemistry.

Blood urea nitrogen (BUN) levels were measured using the i-STAT System (Abbott Laboratories, USA).

Histological Analysis

H&E and Masson's trichrome stains were performed to examine the histopathological changes. To examine the relation calponin expression to sclerotic glomeruli, the number of glomeruli was counted. In each section from animal group stained with Masson's trichrome stain, half of the cortical areas were examined under 400× magnification. Glomeruli with periglomerular calponin expression were grouped as three; >80%, about 50%, <50% periglomerular coverage by calponin-positive.

Antibodies

Primary antibodies for immunohistochemistry; rabbit polyclonal calponin antibody (diluted in 1:200; Millipore, USA), mouse monoclonal nestin antibody (diluted in 1:150; Biogenesis Ltd., UK). Secondary antibodies for immunohistochemistry; peroxidase-conjugated donkey anti-rabbit, and anti-mouse immunoglobulin G (IgG) (diluted in 1:200; Jackson ImmunoResearch Laboratories Inc., USA). Secondary antibodies for confocal microscopy; Alexa Fluor 488-conjugated anti-rabbit IgG antibody (diluted in 1:300; Life Technologies, USA) and Cy3-conjugated anti-mouse IgG antibody (diluted in 1:2,000; Jackson ImmunoResearch Laboratories Inc.).

Immunohistochemistry

Postembedding immunohistochemistry was processed for wax sections (5-μm-thick) after previous method (Lee et al., 2010). Postembedding immunohistochemistry was processed for vibratome sections (50-μm-thick) after previous method (Cha et al., 2001). After immunostaining, the sections were embedded in resin, cut to serial semithin sections (1-μm-thick), and used for serial morphometric analysis.

Immunofluorescent staining was evaluated by confocal laser scanning microscopy (LSM 510 Meta; Zeiss, Germany). Fluorescent images were captured with green laser (excitation 488 nm, emission 490~555 nm) and red laser (excitation 555 nm, emission 505~600 nm) at 1,000 magnification power.

Statistics

Data are expressed as mean±standard deviation (SD). Statistically significant differences between two groups were determined using an unpaired Student's t-test. $p<0.01$ was considered significant.

RESULTS

Laboratory Data of Experimental Models

The BUN levels of ADN rats (both 8 and 12 weeks) were significantly higher than those of sham animals. In contrast, ADN rats (both 8 and 12 weeks) showed significant body weight loss compared with sham animals (Table 1). These laboratory data indicate that adriamicin-treated chronic renal failure was successfully induced.

Histological Examinations

In both 8 weeks- and 12 weeks-ADN rats, typical histo-

Table 1. Changes in serum BUN and body weight levels in sham and ADN rats

	BUN (mg/dL)	Weight (g)
Sham (n=5)	13.1±1.6	492±51
ADN 8 weeks (n=5)	43.4±6.7*	208±15*
ADN 12 weeks (n=5)	48.4±6.5*	204±21*

BUN, blood urea nitrogen; ADN, adriamycin nephropathy.

*$p<0.01$ vs. sham group.

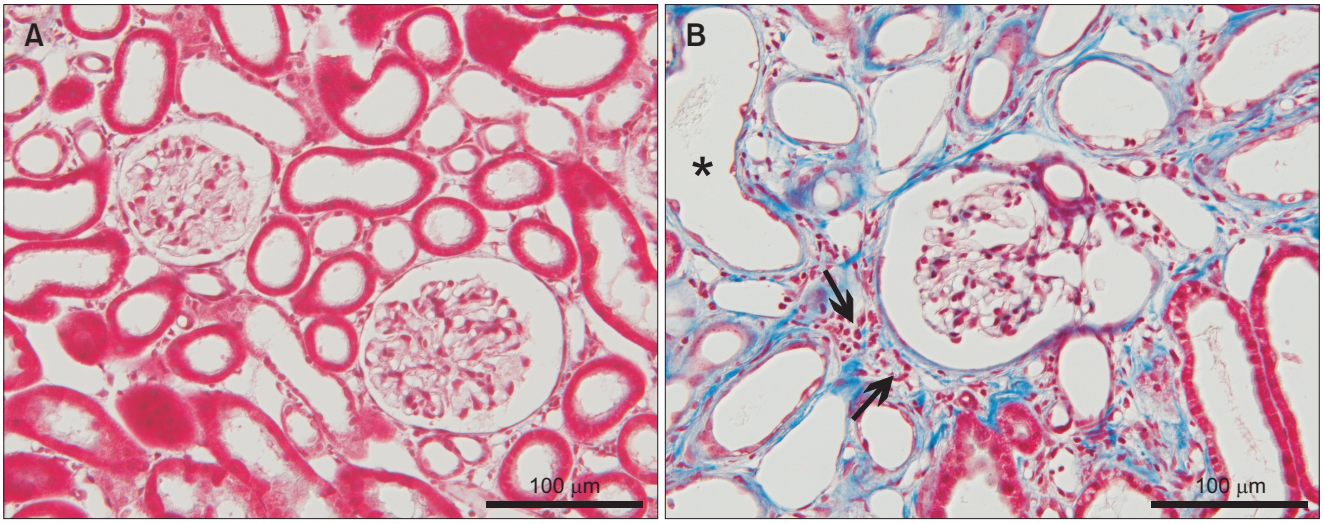

Fig. 1. Light microscopy of a part of renal cortices from sham (A) and adriamycin nephropathy rats (B). (B) Glomeruli show segmental glomerulosclerotic change. Note the expanded tubule (asterisk) and interstitial fibrosis stained blue. Arrows indicate the infiltrated cells. Masson's trichrome stain.

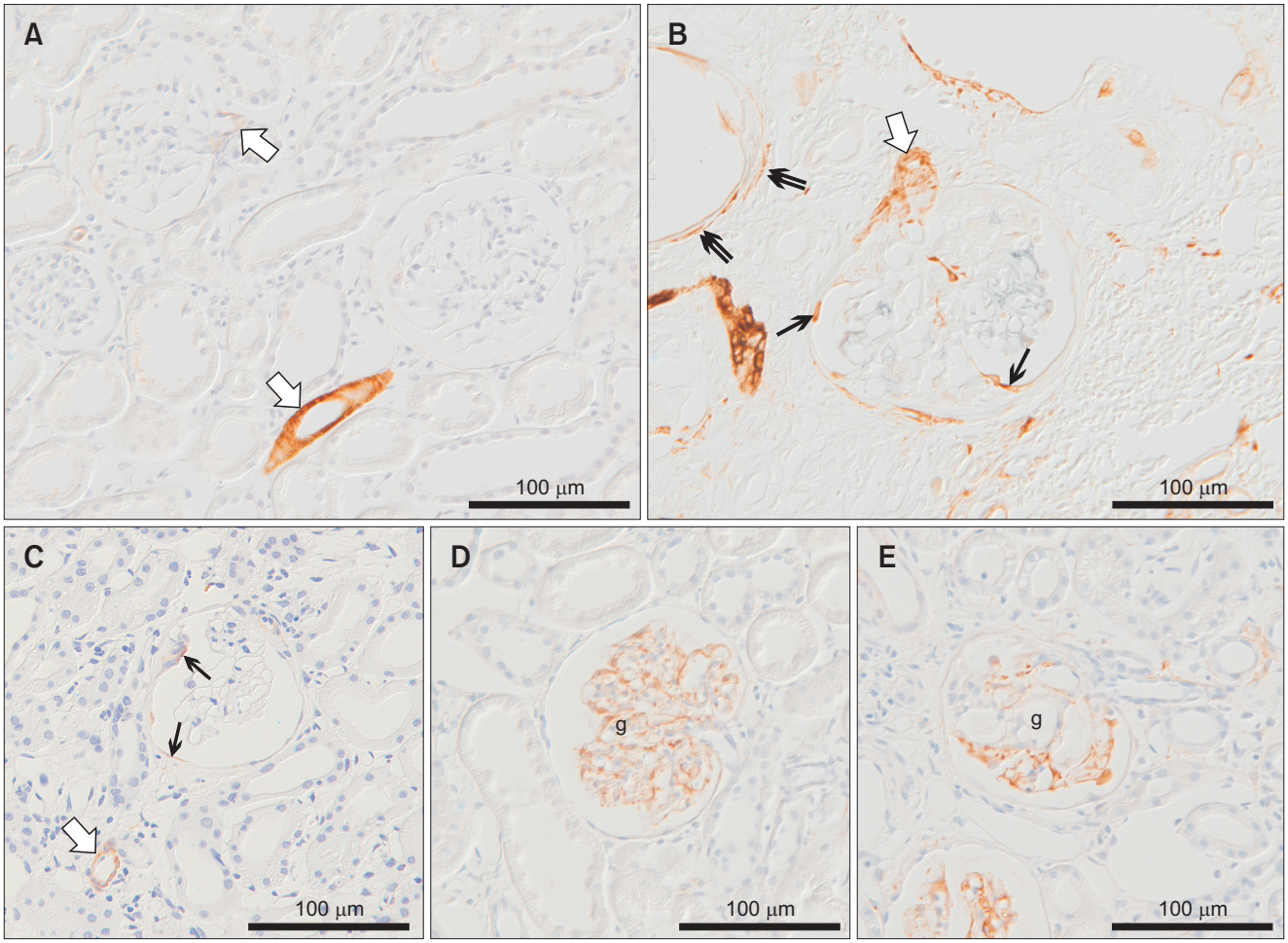

Fig. 2. Light microscopy of a part of renal cortices from sham (A, D) and adriamycin nephropathy (ADN) rats (B, C, E) immunostained with calponin (A, B, C) and nestin (B, D, E) antibodies. Calponin is detected only in renal vessels (A, open arrows), in parietal epithelial cells (PECs; B and C, arrows), periglomerular myofibroblasts (B, double arrows) and vessel (B and C, open arrow). Notice that only PECs (C, arrows) are calponin-positive in some glomeruli. Nestin expression is much reduced in ADN models (compare D with E), note that calponin-positive glomerulus (g) showed low intensity of nestin immunoreactivity in calponin-nestin double immunostained section (B); nestin is blue-colored.

pathological features for chronic renal diseases were demonstrated, such as tubular expansion, tubulointerstitial and periglomerular fibrosis, and mononuclear leukocyte infiltration. Sclerotic glomerular lesions were also evident. The kidneys of sham animals showed no distinct nephritic changes (Fig. 1).

Table 2. Number of glomeruli in chronic renal injury

Group	Glomerular count (n)	Periglomerular coverage by calponin-positive (%)		
		>80	About 50	<30
Adriamycin nephropathy				
8 Weeks	405	4	30	32
12 Weeks	397	10	43	40

Expression of Calponin

In the sham rats, calponin-immunoreactivity was detected only in the smooth muscles of renal vessels (Fig. 2A). In the ADN rats, calponin-immunoreactivity was demonstrated in the periglomerular area as well as in the renal vessels (Fig. 2B). Table 2 showed the numbers of glomeruli with periglomerular calponin-immunopositive cells. In ADN models, 16.3% (8 weeks) and 23.4% (12 weeks) glomeruli were calponin-immunopositive at glomerular area, and there were increases of periglomerular coverage by calponin-immunopositive cells in 12 weeks vs. 8 weeks ADN rats. In nephropathy models, 16.3% (8 weeks) and 23.4% (12 weeks) glomeruli were shown calponin-positive at glomerular area. In all these glomeruli, showing various sclerotic changes, calponin-immunoreactivities were present only both PECs and PMFs (Fig. 2B) except renal vessel. However, in the glomeruli with weak calponin-positive, immunoreactivity was mostly detected in PECs (Fig. 2C).

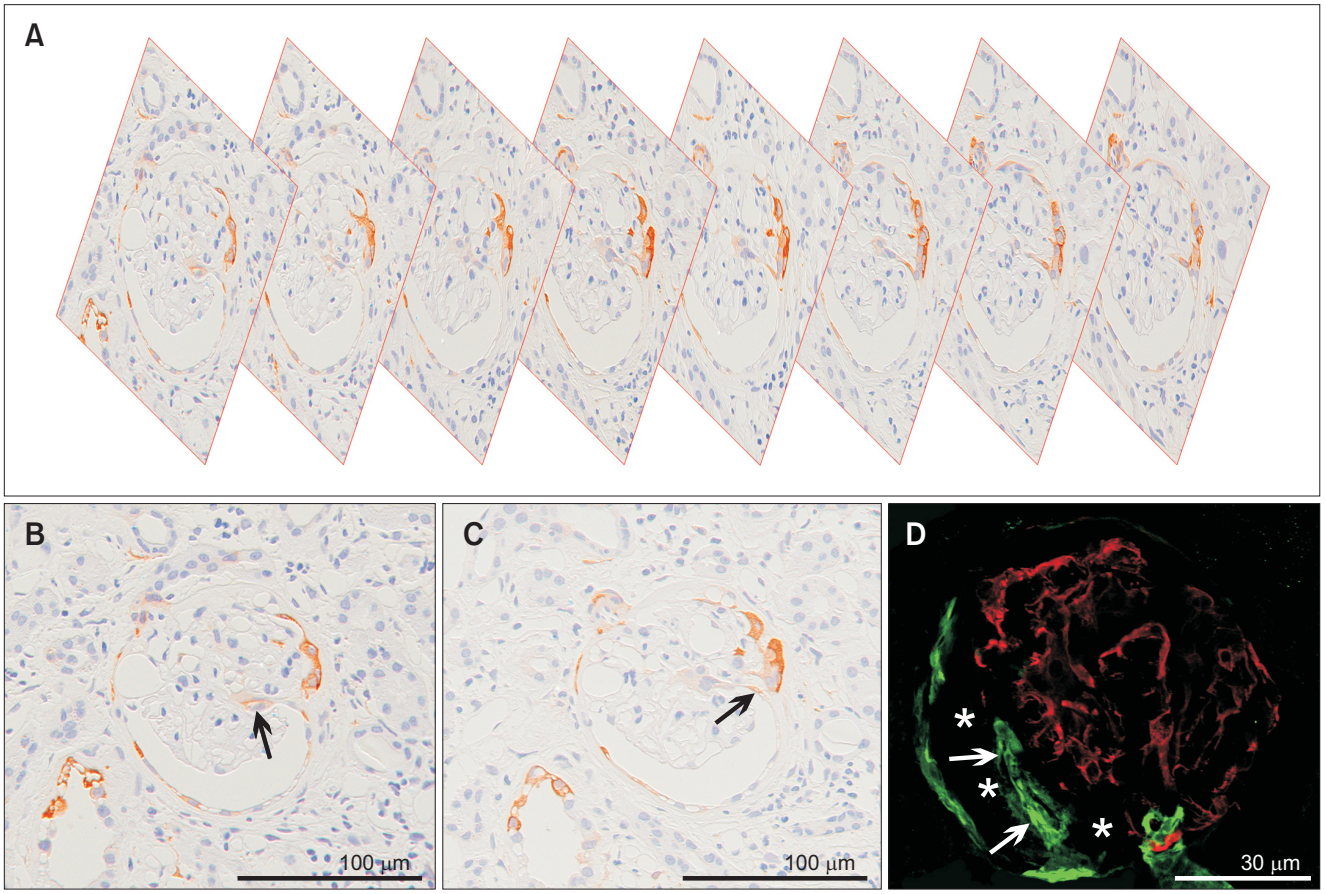

Fig. 3. Representative serial morphometric analysis of adriamycin nephropathy rats. Fig. 3A is eight consecutive semithin-sections used for serial morphometric analysis immunostained using calponin antibody. Fig. 3B is the 1st section of Fig. 3A. In the middle of glomerular tufts, calponin was expressed on the sectional image (arrow). Fig. 3C is the 4th section of Fig. 3A. Note the loop-shaped projection of parietal epithelium into the glomerular tuft (arrow). (D) A merged and overlapped confocal image by five consecutive images showing expressions of the nestin (red) in glomerular tuft and the calponin (green) in parietal epithelial cells. Note the loop-shaped projection of parietal epithelium (arrows) into the glomerular tuft. Asterisks indicate the glomerular tuft region where nestin expression is reduced.

Using double immunohistochemistry, we found that the nestin-immunoreactivity of glomerular tuft was lower in the glomeruli with versus without periglomerular calponin-immunoreactivity (Fig. 2B, D, and E). In the middle of some glomerular tufts, calponin was expressed on the sectional image (Fig. 3B). From the serial morphologic images (Fig. 3A), we identified that such structures were derived from loop-shaped invasion of PECs into the glomerular tuft (Fig. 3C). In addition, nestin expression of glomerular tuft around the such invasions was much reduced (Figs. 2B and 3D).

DISCUSSION

ADN rat model of the present study has been widely used to investigate the progressive nature of chronic renal disease in humans (Lee & Harris, 2011). The laboratory data and general histochemical findings of the present study indicated that animal models were well-established.

Our previous study have shown that calponin-immunoreactivity was localized in periglomerular area of some sclerotic glomeruli from two experimental chronic renal diseases, puromycin aminonucleoside nephropathy and subtotal nephrectomy rats models (Lee et al., 2010). In the present study, we tested whether another chronic renal disease model give the same result, and what detailed morphologic features of periglomerular calponin-positive cells are to investigate the role of calponin during glomerulosclerosis. The calponin expression pattern in periglomerular area of the sclerotic glomeruli was similar to that of our previous study. However, in ADN rats model, PECs as well as PMFs were calponin-positive. Moreover, in the glomeruli with weak calponin-positive, immunoreactivity was mostly detected in PECs. In our previous models, puromycin aminonucleoside nephropathy and subtotal nephrectomy rats models, periglomerular calponin-immunoreactivity was mostly detected in PMFs, and only in some PECs confirmed by observation using seminthin sections (unpublished data). What make this difference is unclear. There are many studies on the glomerulosclerosis using puromycin aminonucleoside/puromycin aminonucleoside nephropathy, and subtotal nephrectomy animal models (Guan et al., 2004; Kelly et al., 2004; Lee et al., 2010). However, there is little report regarding different histopathological findings during glomerulosclerosis among these models. This difference in calponin expression pattern between nephropathy models is need to be further investigated.

The most interesting finding was that some calponin-positive PECs invaded glomerular tuft with loop-shaped projection, and around this projection, nestin expression of glomerular tuft were much reduced. Recently, there is an increasing number of study on PECs, regarded as less-important cells in glomerular function for a long time. Several studies demonstrated their significant contribution to glomerular physiology and numerous glomerular diseases including glomerulosclerosis. It has been reported that PECs play key roles in the development of glomerulosclerosis (Smeets et al., 2004; Ohse et al., 2010; Zhang et al., 2012; Okamoto et al., 2013). In particular, Dijkman et al. (2005) demonstrated that PECs invade the glomerular tuft using three-dimensional reconstruction of serial tissue sections, and concluded that PECs are crucially involved in the pathogenesis of FSGS lesions. One of our result that invasion of PECs into glomerular tuft was very similar with their result. According to present study, calponin was expressed periglomerular area of sclerotic glomeruli only, not of non-sclerotic ones. In addition, PECs, invading into glomerular tuft, expressed calponin. There has been known that in chronic nephropathy glomerular nestin expression is decreased (Liu et al., 2013; Zivotić et al., 2015). These reports agree with our result, in addition, our finding offer an important morphological clue showing that nestin-immunoreactivity is lower in glomeruli with periglomerular calponin-immunoreactivity.

These observations indicate that calponin expression in PECs/PMFs may be closely related to glomerulosclerotic process, although whether it remains unclear that calponin expression is primary or secondary to glomerulosclerotic process.

SUMMARY

Calponin is not expressed normal renal structures except vascular smooth muscle. In sclerotic glomeruli, calponin appears only in the cells located at periglomerular area, PECs and PMFs. Especially, PECs with calponin expression invades glomerular tuft, and nestin expression of the glomerular tuft is much reduced around these invasion region. Therefore, it assumes that calponin is closely related to the glomerulosclerotic process.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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