dendrin, podocalyxin and DAPI was performed. Nuclear dendrin was shown as an image surrounded by podocalyxin and merged with DAPI. There were some clear dendrin-positive nuclei in FSGS (**C**), MN (**F**) and LN (**E**). On the other hand, few dendrin-positive nuclei were present in MCD (**A**). Original magnification was ×400 (for lower magnification), ×1,000 (for higher magnification).

Figure 7: Glomerular expression of nuclear dendrin in several glomerular diseases.

The numbers of dendrin-positive nuclei per glomerulus were statistically significant for FSGS, MN and LN. Asterisks show that the difference in the ratio, compared with the controls, was significant ($p<0.05$). The columns represent the mean ± SE of each value from different samples.

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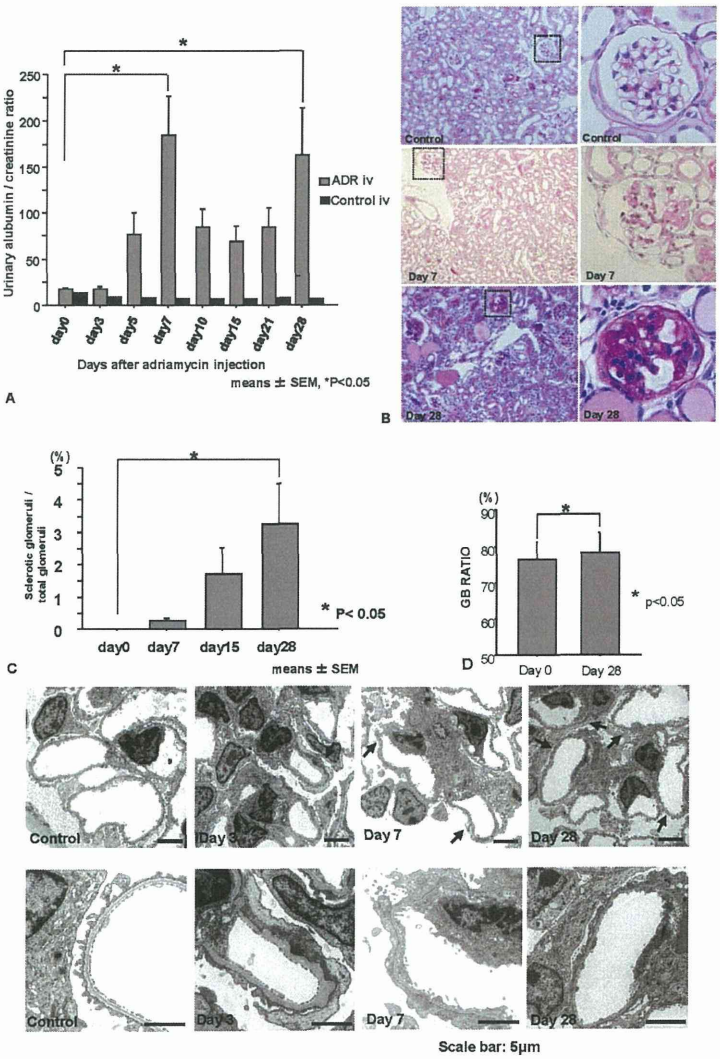

Figure 1

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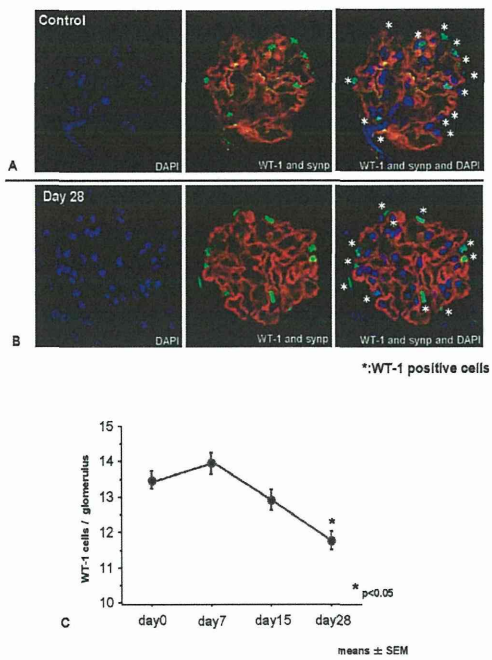

Figure 2

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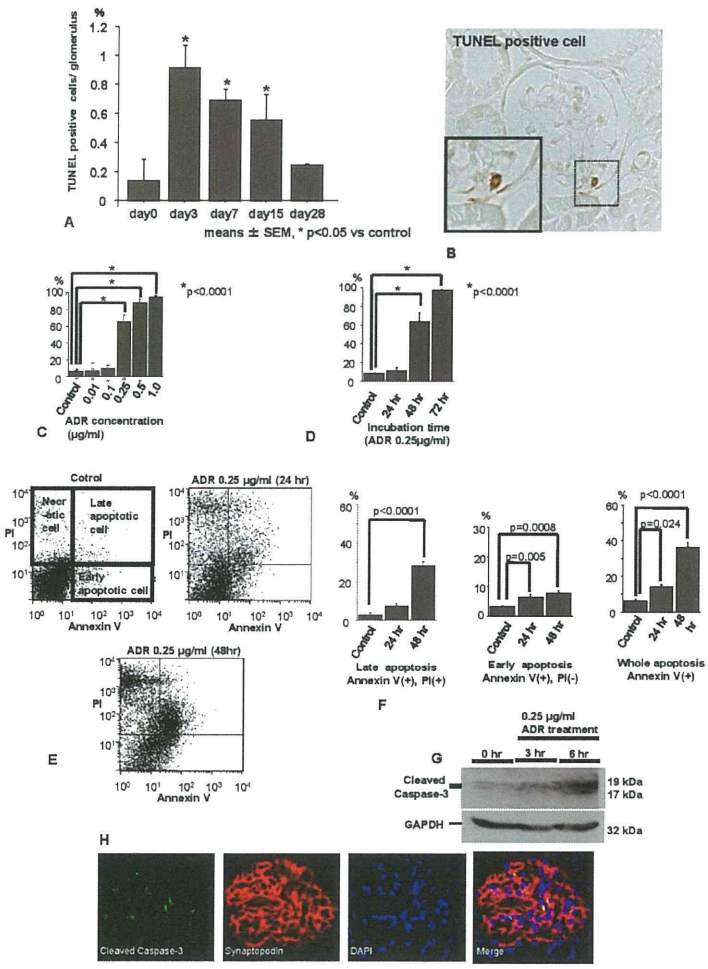

Figure 3

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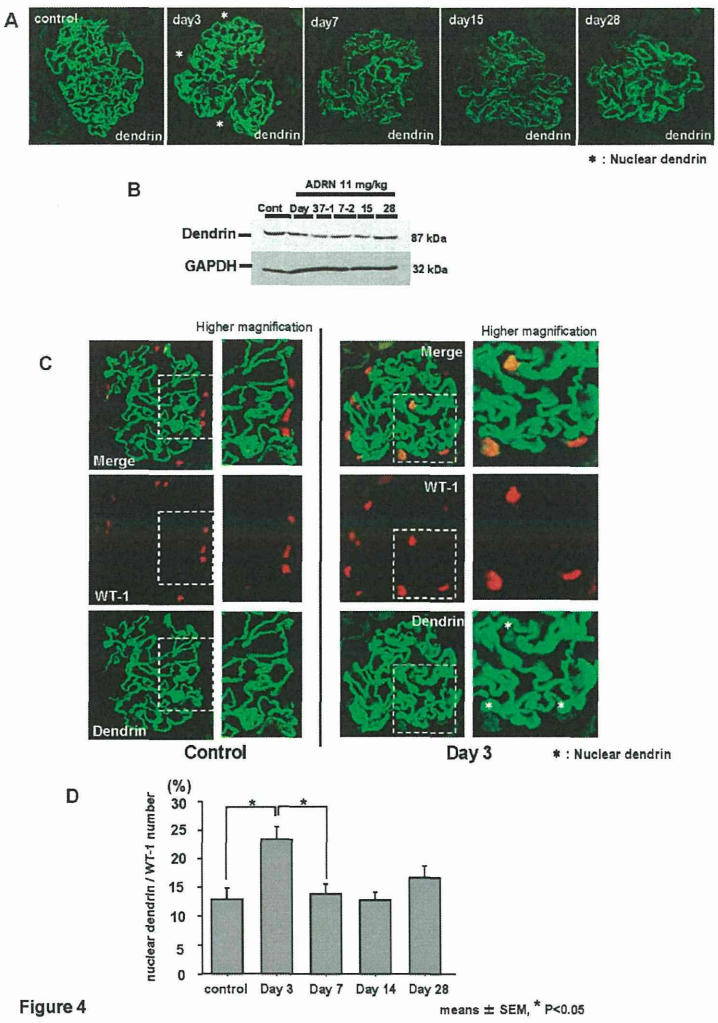

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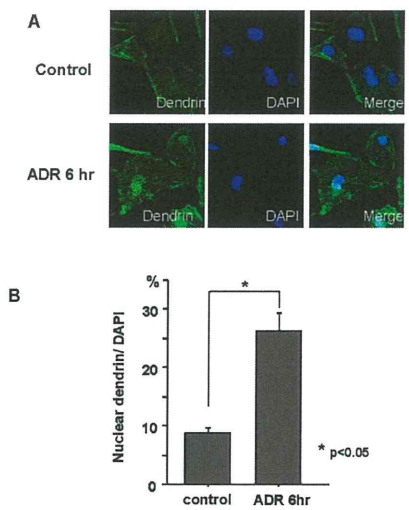

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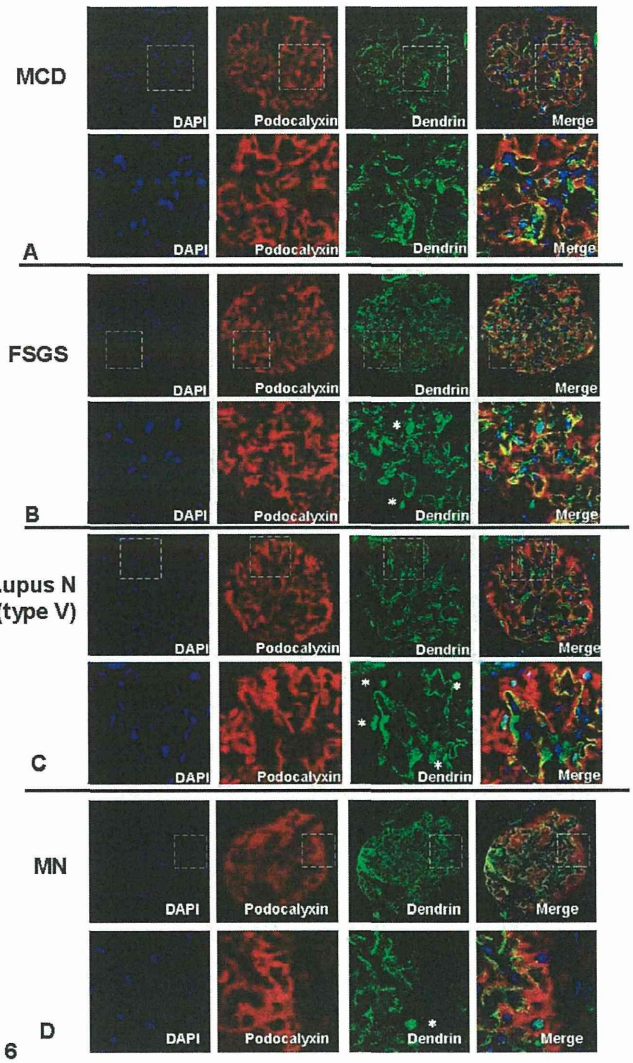

Figure 6

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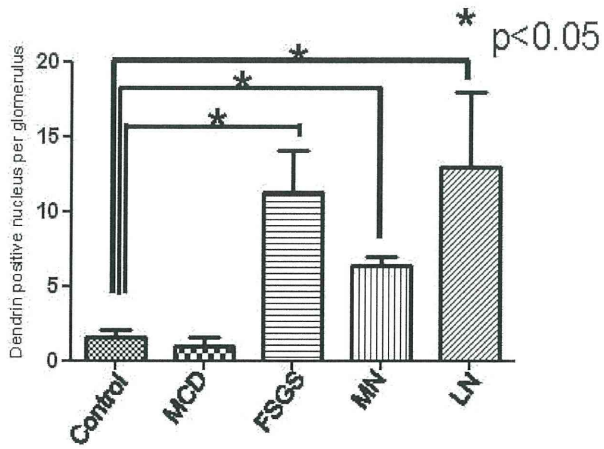

Figure 7

170x245mm (73 x 77 DPI)

Dendrin location in podocytes is associated with the disease progression in animal and human glomerulopathy

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Short title: Nuclear dendrin in in mouse and human glomerulopathy

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Keywords: dendrin, podocyte, ADR mice, glomeruloscrelosis, nephrosis, apoptosis

Number of words: 3448

Conflict of interest statement: *None declared.*

Abstract

Background: ADR nephrosis in mice has been extensively studied and has enabled a greater understanding of the processes underlying the progression of renal injury. Dendrin is a novel component of the slit diaphragm (SD) with proapoptotic signaling properties, and it accumulates in the podocyte nucleus in response to glomerular injury in mice. The present study re-evaluated chronic progressive nephropathy in ADR mice and the localization of dendrin in mice and in human glomerulopathy. **Methods:** To investigate the localization of dendrin, a mouse model for nephrosis and glomerulosclerosis was used, in which ADR was injected once. WT-1 positive cells and apoptotic cells were counted *in vivo and in vitro*. To check the expression of dendrin in ADR mice, immunostaining and western blot were performed. A survey of dendrin staining was performed on human kidney biopsy specimens. **Results:** The injection of ADR induced proteinuria, podocyte loss and glomerulosclerosis. It also caused the relocation of dendrin from the SD to the podocyte nucleus. We demonstrated the location of dendrin to podocyte nuclei in several human glomerulopathy. The mean occurrence of dendrin-positive nucleus per glomerulus increased in several cases of human glomerulopathy. **Conclusions.** These findings suggest that the relocation of dendrin to the podocyte nuclei is useful as a novel marker of podocyte injury in human glomerulopathy.

Introduction

Podocytes are highly specialized, terminally differentiated epithelial cells that do not show characteristic cell division and proliferation [1]. Podocytes serve as the final barrier to urinary protein loss through the special formation and maintenance of foot processes (FPs) and interposed slit diaphragm (SD) [2]. All forms of nephrotic syndrome are characterized by abnormalities in podocytes, including effacement of FPs and/or molecular reorganization of the SD [1-3]. Defects in podocyte structure, function or number can lead to pathologic lesions, known as focal segmental glomerulosclerosis (FSGS) [4-6]. In rat puromycin aminonucleoside (PAN) nephrosis, injured podocytes are manifested by the loss of interdigitating FPs [7][8], detachment from the glomerular basement membrane (GBM), and associated leakiness of the glomerular filter, resulting in proteinuria [6,7]. A reduction in podocyte numbers directly causes proteinuria and leads to glomerulosclerosis [4,9,9-11]. Several groups have shown that apoptosis is a major cause of podocyte loss from the GBM [4], leading to proteinuria and/or glomerulosclerosis in rat PAN nephrosis [6,11] and in human diabetic nephropathy [12] and IgA nephropathy[9,13].

Adriamycin (ADR) nephrosis is well known as a nephrosis and FSGS model in rats [14,15]. An LPS-induced nephrosis model has been established in mice. The model shows transient proteinuria, but no glomerulosclerosis [16]. The first description of ADR including renal injury was in 1998 in mice [17]. Since then, ADR nephrosis in

mice has been extensively studied and has enabled a greater understanding of the processes underlying the progression of renal injury [18]. To elucidate the mechanism of podocyte injury and podocyte loss from the GBM, it is necessary to re-evaluate podocyte apoptosis, podocyte loss and glomerulosclerosis in the ADR mice during a set time course.

Dendrin is a proline-rich protein of unknown function that was originally identified in telencephalic dendrites of sleep-deprived rats [19]. In the brain, two protein variants (81 kDa, 89kDa) have been identified in cytosolic and membranous protein fractions [19]. Recently, dendrin has been seen as a component of the slit diaphragm (SD) complex that relocates to the nucleus of injured podocytes in a murine model of crescentic glomerulonephritis [20]. Also, TGF-β is now known to promote the nuclear translocation of dendrin, and nuclear dendrin is known to amplify TGF-β-induced podocyte apoptosis. Dunner *et al.* reported that dendrin forms a linear pattern on the epithelial side of the glomerular capillary loops, corresponding to the podocytes of normal humans and minimal change disease (MCD) patients [21]. However, nuclear dendrin was not found in the kidneys of patients with MCD.

In the present study, podocyte apoptosis, podocyte loss and glomeruloscrelosis were re-evaluated in a murine model of chronic progressive nephropathy with significant and persistent proteinuria using ADR. Moreover, the localization of dendrin in model mice and in human glomerular diseases was examined.

Methods

Mouse model of ADR-induced proteinuria

Female BALB/c mice weighing about 20 g and aged 8 weeks were obtained for the induction of ADR nephropathy from Oriental Yeast Co., Ltd. (Tokyo, Japan). ADR (doxorubicin hydrochloride; Wako, Osaka, Japan) diluted with 0.9% saline at a dose of 11 mg/kg body weight (BW) was injected once via the tail vein of each non-anesthetized mouse. Age-matched control female BALB/c mice were injected with an equal volume of PBS only. All mice were weighed and urine samples were collected once every few days. Urinary albumin/creatinine ratio (ACR) was measured using an immunoassay (DCA 2000 Systems; Siemens Medical Solutions Diagnostics, Tarrytown, NY) with a Bayer DCA 2000+ chemical analyzer (Bayer Diagnostics, Elkhart, IN) [22]. After anesthesia with sodium pentobarbital (100 mg/kg body weight; Dainippon Sumitomo Pharma, Osaka, Japan), five mice per day were euthanized on days 3, 7, 14, and 28 after the injection of ADR. All mice were housed under specific pathogen-free conditions using standard animal cages with free access to standard chow and drinking water.

Antibodies

Polyclonal rabbit anti-WT-1 antibody (Santa-Cruz Biotechnology Inc, CA), monoclonal mouse anti-WT-1 antibody (Dako Corporation, CA), monoclonal mouse

anti-GAPDH antibody (Abcam, Cambridge, UK), and polyclonal rabbit anti-cleaved caspase-3 antibody (Cell Signaling, Danvers, MA) were purchased for immunohistochemistry and WB analysis. Antibody against podocalyxin was a gift from Denka Seiken CO., LTD. To generate antibody against human dendrin, rabbits were immunized with a hemocyanin-conjugated peptide (single letter code, RHSQTLPRPWAPGGTGWC) corresponding to the amino acids of human dendrin. The anti-serum was affinity-purified with the corresponding peptide. Rabbit polyclonal antibodies against dendrin [20] and synaptopodin [23] have been described previously.

Plasmid constructs

Human dendrin cDNA (OriGene Technologies, Inc, MD) was cloned into pEGFP-C1 (BD Biosciences Clontech) and verified by DNA sequencing. FLAG- and GFP-tagged rat dendrin constructs have been described previously [20].

Renal histology and Immunohistochemistry

For the histological study, the mouse kidneys were fixed by perfusion fixation using 4% paraformaldehyde (PFA). The fixed kidneys were embedded in paraffin for light microscopy (LM) study. The 3 μm-thick sections were stained using the periodic acid-Schiff (PAS) technique. The sections were observed under LM (Olympus BX41;

Olympus). In PAS stained light microscopic sections, at least 100 midsections of glomerular areas and Bowman's capsule areas were measured using a digitizer KS-400 Imaging System as described previously [24].

For electron microscopy (EM), after perfusion fixation with 4% PFA, small blocks of kidney were fixed with 2% glutaraldehyde and postfixed in 1% osmium tetroxide (OsO_4). The samples were dehydrated in graded ethanol and embedded in epoxy resin. Ultrathin sections (80-100 nm thick) were stained with uranyl acetate and lead citrate and examined by electron microscopy (H-7100; Hitachi, Tokyo, Japan) at 75 kV.

For the immunofluorescence study, kidneys were fixed with the same agents as for LM, placed in OCT compound, frozen in liquid nitrogen and stored at -80 ℃. Immunostaining of podocyte-associated molecules (dendrin, WT-1 and synaptopodin) was performed. Double-stained immunofluorescence studies of the podocyte-associated proteins and post-staining with DAPI were performed as described previously [20]. The sections were observed under a confocal laser microscope (Olympus FV1000; Olympus, Tokyo, Japan). To examine the number of podocytes in glomeruli, double-positive cells in WT-1 and DAPI staining were counted in more than 100 glomeruli.

Human tissue samples