Newly Identified Molecules Related to Podocyte Injury Induced by Adriamycin

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Objective: Podocytes play an important role in maintaining the structural integrity and function of the glomerular filtration barrier. Adriamycin (ADR) – induced podocyte injury has been extensively studied, however, its mechanism remains unclear. In the present study, we aimed to explore novel genes associated with podocyte injury induced by ADR.

Methods: A microarray assay was carried out on isolated podocytes with and without ADR treatment and analyzed using Genespring GX software. Two genes, Fos-like antigen 1 (FOSL1) and Regulator of G-protein signaling 2 (RGS2) were selected as candidate genes because their levels showed the most significant changes after ADR-induced podocyte injury. To confirm the changes in mRNA levels and their protein expression, ADR was added to cultured mouse podocytes and analyzed at 1, 2, 6, and 24 hours using real-time PCR and immunofluorescence. Finally, we also confirmed the expression of both proteins in vivo, by staining mouse kidney tissue at 0, 3, and 15 days after ADR injection.

Results: Microarray data showed that after ADR treatment, FOSL1 was up-regulated, while RGS2 was down-regulated. Real-time PCR analysis confirmed that ADR induced a significant increase of FOSL1 at 24 hours (p<0.001, 24 h vs. control), and a marked down-regulation of RGS2 (p<0.001, at each time point vs. control). Immunofluorescence analysis of podocytes treated with ADR for 24 hours showed an up-regulation tendency for FOSL1 protein and a down-regulation tendency for RGS2. On ADR-induced injury mice specimens, the expression of FOSL1 was increased in a time dependent manner after the injection; on the other hand, RGS2 was down-regulated, especially 15 days after injection.

Conclusion: FOSL1 and RGS2 were both expressed in podocytes, and were significantly regulated after ADR treatment, revealing a possible role in podocyte injury induced by ADR. These results could help guide research further to finally elucidate the underlying mechanisms in podocyte injury.

Key words: FOSL1, RGS2, podocytes, ADR-induced injury

Introduction

Podocytes are highly specialized, terminally differentiated epithelial cells located outside of the glomerular basal membrane (GBM), with vascular endothelial cells being inside, and together they serve as the final barrier for preventing proteinuria. The disruption of podocyte structure or function may eventually result in attenuation of renal function accompanied by podocyte foot processes effacement, podocyte loss, and glomerulosclerosis. These pathological changes in podocytes are well documented in human glomerular diseases such as focal segmental glomerulosclerosis (FSGS) and diabetic nephropathy.

Adriamycin (ADR) has been widely used in rodents for inducing renal injury analogous to human FSGS. As an experimental model, injecting ADR into BALB/c mice results in proteinuria, elevated serum creatinine, and reduced creatinine...
clearance. Kidney histological changes include foot-process effacement and detachment from the GBM, glomerulosclerosis, and tubule-interstitial inflammation. In addition, treatment with ADR causes actin filament reassembly and apoptosis in the cultured podocytes. The downstream signaling in ADR injured podocytes is still unclear.

In the present study, we used a microarray assay to help explore novel genes associated with podocyte injury induced by ADR. We report herein the identification of two novel molecules, FOSL1 and RGS2, that show regulation changes associated with podocyte injury induced by ADR.

Methods

1. Cell culture and ADR treatment
Conditionally immortalized mouse podocytes were cultured as described previously. Briefly, undifferentiated podocytes were cultured in a RPMI1640 medium (Sigma-Aldrich, Tokyo, Japan) with 10% FBS, 100 units/ml Pen/Strep (Life Technologies, USA), and γ-IFN 10U/ml at 33°C. For differentiation, podocytes were shifted to nonpermissive conditions at 37°C in the absence of γ-IFN and grown for 7-14 days. To detect the reaction to ADR, 0.25 μg/ml ADR was added to the differentiated podocytes for indicated periods in the regular medium.

2. Microarray assay
A microarray assay was designed to examine the effects of ADR on the cultured podocytes as follows: (1) ADR groups, differentiated podocytes treated with 0.25 μg/ml ADR for 1, 2, 6, and 24 hours; and (2) control group, differentiated podocytes without ADR treatment. Total RNA was extracted from both groups. cDNA was synthesized from the total RNA and a microarray assay was carried out for each time point by Bio Matrix Research (BMR) (Technical Development Center, Chiba, Japan). Microarray data was analyzed using Genespring GX software (Agilent).

3. Real-time PCR
Total RNA was extracted from cultured podocytes with or without ADR treatment at the specified time points using an RNeasy Mini Kit (Qiagen, Germany). cDNA was synthesized from 1 μg total RNA using Random Decamers (Ambion, Austin, TX, USA), and reverse transcriptase, M-MLV (Invitrogen Life Technologies, Carlsbad, CA). Real-time PCR was performed using TaqMan Fast Advanced Master Assay fos-like antigen 1 (FOSL1, Assay ID: Mm04207958_m1), regulator of G-protein signaling2 (RGS2, Assay ID: Mm00501385_m1), glyceraldehydes-3-phosphate dehydrogenase (GAPDH, Assay ID: Mm99999915_g1), and a 7500 real-time PCR system (Applied Biosystems, Life Technologies) following the manufacturer’s instructions. All measured values were normalized with GAPDH and calculated using the comparative CT (ΔCT) study.

4. Immunofluorescence
To check the localization of FOSL1 and RGS2, the differentiated podocytes were cultured on collagen type I coated cover glass and treated using ADR in a 0.25 μg/ml concentration for 24 hours, and then fixed with 2% PFA and 4% sucrose in PBS. Fixed cells were permeabilized with 0.3% Triton in PBS. After a blocking solution (2% FCS, 2% BSA, 0.2% fish gelatin in PBS), cells were incubated at 4°C with the first antibodies, as follows: anti-FOSL1 antibody (rabbit polyclonal antibody, Abbiotec, LLC, San Diego, CA, 1:100) and anti-RGS2 antibody (rabbit polyclonal antibody, Sigma-Aldrich, Saint Louis, MO, 1:50). Finally, cells were incubated with donkey anti-rabbit IgG conjugated with Alexa 488 as a second antibody. DAPI was used as a nuclei marker. To measure the podocyte injury induced by ADR, we performed triplicate staining using Alexa Fluo555-phalloidin (for actin filament, Invitrogen, CA, 1:250), anti-synaptopodin antibody (mouse monoclonal antibody, Progen, Heidelberg, 1:10) and DAPI. All the images were captured using a confocal laser microscope (Olympus FV1000, Tokyo, Japan).

5. Western blotting
To evaluate the protein expression in cultured podocytes with and without ADR treatment, whole-cell extract samples were prepared using 1% Triton X-100 lysis buffer with Complete Mini protease inhibitor (Roche, Germany) and Phostop phosphatase inhibitor (Roche, Germany). Protein amounts were measured with the Pierce 660 nm protein assay (Thermo Scientific) using albumin (Thermo Scientific) as a standard. Whole-cell lysate were separated
by SDS-PAGE, transferred onto a PVDF membrane (Millipore), and subjected to the specific antibody: Rabbit anti-FOSL1 (1:1000), Rabbit anti-RGS2 antibody (1:800) or Mouse anti-synaptopodin (1:100). Mouse anti-GAPDH antibody (mouse monoclonal antibody, Sigma–Aldrich, Saint Louis, MO, 1:10000) was used as an internal control. The Supersignal Western Dura reagent (Thermo Scientific) was used for detection, and LAS4000 was used to visualize the band.

6. ADR-nephrosis mice model and immunofluorescence of these specimens

This study was conducted according to the Declaration of Helsinki and was approved by Institutional Review Board of Juntendo University. Female BALB/c mice were purchased from Oriental Yeast Co., Ltd. (Tokyo, Japan). ADR (doxorubicin hydrochloride; Wako, Osaka, Japan)–induced nephropathy was conducted. In brief, ADR diluted with 0.9% saline at a dose of 11 mg/kg body weight was injected once into BALB/c mice at 8 weeks of age via the tail vein. Age-matched control mice were injected with an equal volume of PBS (pH 7.0). On the indicated days, mice were sacrificed and kidney tissues were obtained after PFA-perfusion.

To detect FOSL1 and RGS2 expression in ADR-nephrosis mice, the mice kidneys were perfusion-fixed by 4%PFA and 20% sucrose in PBS, placed in an OCT compound, frozen in liquid nitrogen, and then stored at −80°C. 5 μm–thick sections were incubated with an anti-FOSL1 antibody (1:100) or anti-RGS2 antibody (1:50) at 4°C overnight, and double staining of Synaptopodin and post-staining of DAPI for nuclei were performed subsequently.

7. Statistical analysis

All statistical analyses were carried out using SPSS, Version17.0. Data were represented as the mean ± SE. A student’s t-test or ANOVA was used to analyze the differences between groups. p < 0.05 was regarded as statistically significant.

Results

1. Microarray assay results from cultured podocytes showed that FOSL1 and RGS2 are highly regulated after ADR treatment

To explore new molecules associated with ADR-induced podocyte injury, we analyzed cultured podocytes before and after ADR treatment. Using a microarray assay we compared alterations of gene expressions in podocytes without ADR treatment, and in podocytes treated for 1, 2, 6, and 24 hours with 0.25 μg/ml of ADR. 423 genes were up-regulated and 332 were down-regulated in all of the ADR groups. All of these genes had expression changes greater than 2 fold when compared with the non–treated podocytes (control group). Among these genes profiles, we chose FOSL1 with a significant increase and RGS2 with a significant decrease in ADR groups as candidate genes (unpublished observation).

2. mRNA expression of selected genes in ADR-induced podocyte injury

To confirm the results from the microarray assay,
Figure 2  ADR induced podocyte injury in vitro
A. Triplicate staining of Phalloidin (red, for actin filament staining), Synaptopodin (green, a podocyte marker), and DAPI (blue, for nuclei staining) was performed on podocytes with or without ADR administration. After 24 hours, ADR treated podocytes showed decreased stress fiber, a rearrangement of actin fiber, and decreased synaptopodin. B. Western blot result showed that the protein expression level of synaptopodin was decreased at 24 hours after ADR treatment. C. Quantification of synaptopodin was represented as the protein expression ratio normalized to GAPDH. N=1 experiment.

Figure 3  ADR regulated FOSL1 and RGS2 protein expressions in cultured podocytes
A. FOSL1 protein localized especially at the nuclei, and after ADR treatment, the distribution was changed so that it stained homogeneously both at the cytoplasm and the nuclei. B. RGS2 protein was distributed in the cytoplasm and nuclei of podocytes. After 24 hours’ exposure to ADR, a slight reduction in the distribution in the cytoplasm was observed. C. Using western blotting, FOSL1 was not changed significantly in ADR-induced podocyte injury. Using H2O2 (100 μM), a previously established FOSL1-inducer, podocyte expression of FOSL1 increased after 3 hour treatment. Jurkat cells lysate was used as a positive control. D. RGS2 was down-regulated at 6 and 24 hours after ADR treatment in western blot study. Mouse heart sample was used as a positive control. Quantifications of FOSL1 (E) and RGS2 (F) were represented as the protein expression ratio normalized to GAPDH. N=1 experiment.
we used real-time PCR to detect the mRNA levels of the selected genes. For this experiment we used differentiated podocytes treated with 0.25 μg/ml of ADR and analyzed them at 0, 1, 2, 6 and 24 hours after treatment.

The expression pattern of FOSL1 mRNA was different from the results observed in the microarray data. Treatment with ADR for 24 hours increased the mRNA levels of FOSL1 in a time-dependent manner (p < 0.05 at 2 hours vs. control, p < 0.01 at 6 hours and p < 0.001 24 hours vs. control) (Figure-1A). RGS2 mRNA levels showed a similar pattern to the results obtained in the microarray assay. The mRNA levels were significantly reduced at each time point (1, 2, 6, and 24 hours) after ADR treatment (p < 0.001, compared with the control) (Figure-1B).

3. ADR induced each of these protein expression changes in the cultured podocytes

To detect the expression of FOSL1 and RGS2 in the cultured podocytes with and without ADR treatment, we performed immunofluorescence staining. Triplicate staining of phalloidin (red), synaptopodin (green), and DAPI (blue) was carried out on podocytes treated with 0.25 μg/ml ADR for 24 hours and also for podocytes without ADR treatment. Decreased stress fiber, rearrangement of actin fiber and decreased expression of synaptopodin were observed in ADR treated podocytes, indicating podocyte injury (Figure-2A). Western blot results showed that the protein expression level of synaptopodin was decreased at 24 hours after ADR treatment (Figure-2B, C).

FOSL1 is localized especially in the podocyte nuclei in normal conditions, however after ADR treatment, an increase in the cytoplasm and the nuclei in its immunofluorescence intensity was confirmed (Figure-3A). RGS2 protein was distributed in the cytoplasm and nuclei of podocytes before treatment. However, only a slight reduction was observed in the cytoplasm after ADR treatment (Figure-3B).

We analyzed the FOSL1 and RGS2 protein expression by western blotting methods. FOSL1 was not changed significantly in ADR-induced podocyte injury, while after 3 hours’ treatment of 100 μM H2O2, a well-used FOSL1-inducer, FOSL1 expression was increased (Figure-3C, E). RGS2 was down-regulated at 6 and 24 hours after ADR treatment (Figure-3D, F)

4. FOSL1, RGS2 expression in ADR-nephrosis mice

To detect FOSL1 and RGS2 expression in vivo, immunofluorescence was carried out on ADR-nephrosis mice kidney samples. Synaptopodin, a

![Figure 4](image-url)
specific podocyte marker, was observed as a capillary pattern. FOSL1 was expressed along synaptopodin and perinucleus before ADR injection. Following ADR-induced nephrosis, FOSL1 expression in cytoplasm was increased and a significant translocation to nuclei was observed (Figure-4A). RGS2 expression was decreased in a time-dependent manner after ADR injection (Figure-4B).

Discussion

In this study, we found two genes (FOSL1 and RGS2) that were regulated significantly in ADR-induced injured podocytes, we confirmed the mRNA expressions by real-time PCR, and finally showed evidence of the proteins’ expression both in vivo and in vitro by immunofluorescence.

FOSL1, also known as Fra-1, is a member of FOS family that includes c-FOS, FOSB, and FOSL2. Similar to other members, FOSL1 protein that, together with Jun family members (c-Jun, Jun-B and Jun-D), composes the transcription factor activator protein-1 (AP-1). There is repeated evidence suggesting that AP-1 participates in the molecular mechanisms of cell proliferation, differentiation, apoptosis, and transformation. In addition, the changes of the transcriptional ability of AP-1 are not yet well understood. The activation of AP-1 may increase the mRNA level of endothelin-1, causing the rearrangement of F-actin in podocytes. As a member of AP-1 complex, FOSL1 disordered actin filament formation through down-regulating RhoA-GTP activity in colon carcinoma cells. In our work, in ADR-induced injured podocytes, F-actin reorganization was observed. A single nucleotide polymorphisms (SNP) study in immunoglobulin A (IgA) nephropathy patients suggested that SNP of FOSL1 may contribute to the development of foot-process effacement. These results suggest that the up-regulation of FOSL1 in podocytes may increase RhoA-GTP activity and protect from podocyte detachment.

RGS2, a regulator of G protein signaling 2, is a member of the regulator of G protein signaling (RGS) family that consists of more than 20 identified proteins and serve as GTPase-activating proteins. RGS2 selectively inhibits Gαq and Gαi signaling and their respective downstream signals. In the kidneys, it has been reported that RGS2 expresses in glomerular mesangial cells (GMCs) and tubular cells, including proximal and distal tubules and collecting ducts. In this study, we showed RGS2 expression in podocytes, as well as immunofluorescence–displayed RGS2 localization in the cytoplasm and nuclei of podocytes. However, there are some different results about RGS2 location in nuclei in COS-7 cells and human astrocyte 1321N1 cell. We also showed a reduced level of RGS2 in ADR-treated injured podocytes. Some researchers have demonstrated different affects of RGS2 deficiency, including hypertension and accelerated kidney fibrosis following unilateral ureteral obstruction in mice. A siRNA–mediated knockdown of RGS2 increases mouse urotensin-II–induced [Ca2++]i elevation and contraction in murine GMCs. Another study revealed that the down-regulation of RGS2 is neuroprotective in Huntington’s disease. Taken together, our results showing the reduction of RGS2 in ADR-treated injured podocytes provide new insights about the kidney disease, especially for nephrosis and glomerulosclerosis.

In conclusion, our findings identified FOSL1 and RGS2 were significantly regulated and we can speculate they play a specific role in ADR-induced podocyte injury. Further research is needed to elucidate the underlying mechanisms of the molecules in the process of podocyte injury.

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