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A novel method for isolating podocytes using magnetic-activated cell sorting

Ayumi Murakami1, Hisashi Oshiro1, Seiichi Kanzaki1, Akira Yamaguchi1, Shoji Yamanaka1, Mitsuko Furuya1, Satoshi Miura2, Hiroshi Kanno3, Yoji Nagashima1, Ichiro Aoki1 and Kiyotaka Nagahama1

1Department of Pathology, 2RI Research Center and 3Department of Neurosurgery, Yokohama City University School of Medicine 3-9 Fukuura, Kanazawa-ku, Yokohama 236-0004 Japan

Correspondence and offprint requests to: Kiyotaka Nagahama; E-mail: nag_k@med.yokohama-cu.ac.jp.
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Abstract

Background. A large body of accumulated data has now revealed that podocytes play a major role in the development of proteinuria. However, the mechanisms of podocyte injury, leading to foot process effacement and proteinuria, are still unclear partly due to the current lack of an appro-
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Methods. After endothelial cell depletion using anti-CD31 antibody, nephrin-positive cells were prepared from mouse kidneys using magnetic activated cell sorting with polyclonal rabbit anti-nephrin antibody. Purity of the positively sorted cells was determined by confocal microscopy and fluorescence-activated cell sorting (FACS) analysis. Expression profiles of podocyte-specific molecules in the sorted fractions were characterized by qualitative PCR and immunoblot analysis.

Results. Nephrin-positive cells, isolated from mouse kidneys within 6 h, showed dual positivity for synaptopodin and rabbit IgG on confocal microscopy. FACS analysis revealed that the purity of the positively sorted fractions was ~75%. The nephrin-positive cells sorted by this approach showed a significantly higher expression of podocyte-specific molecules compared with nephrin-negative fractions.

Conclusions. These data strongly suggest that our novel method for isolating podocytes has great utility for various downstream applications such as genomic analysis, proteomics and transcriptomics to elucidate molecular profiling of podocyte biology in vivo compared with conventional methods as our approach requires only several hours to complete and no tissue culture.

Keywords: MACS; nephrin; podocyte isolation

Introduction

Various methods have been established to date to isolate podocytes to define their critical role in glomerular dysfunction. In general, glomeruli are purified by conventional sieving procedures for preparing homogenous populations of cells by utilizing their unique surface molecules and has been widely employed in various research fields including immunology, neurology and stem cell research.

Materials and methods

Anti-nephrin antibodies

Establishing a novel approach for separating podocytes using the MACS system requires a large amount of antibodies; thus, we generated anti-nephrin antibodies using rabbit as previously described [9]. In brief, a recombinant protein containing a part of the intracytoplasmic region corresponding to amino acids 1099–1177 or extracellular region corresponding to amino acids 768–832 was fused to either GST- or MBP-tagged protein and produced in Escherichia coli BL21 cells. The GST-fused antigen was used to immunize rabbits (Japan SLC Co., Shizuoka, Japan), and antisera was then collected to purify rabbit IgG using immunoaffinity columns coupled with the MBP-tagged protein. Biotin labelling of anti-nephrin antibody was performed using EZ-Link Sulfo-NHS-LC-Biotin Reagents (Thermo Fisher Scientific, Waltham, MA, USA) according to manufacturer’s instruction.

Podocyte isolation

Kidneys from female B6 mice at 8 weeks of age (Japan SLC, Shizuoka, Japan) were minced into small pieces with two scalpels, and then digested with 2 mg/mL of collagenase (Wako, Osaka, Japan) in complete medium (RPMI-1640 containing penicillin, streptomycin and 5% FCS) at 37°C for 40 min with mild rotation. The specimens were then passed through a 100-μm cell strainer (BD Biosciences, San Jose, CA, USA) with a flattened pestle, and then treated with ACK lysis buffer to remove the red blood cells. After washing with complete medium, further digestion was performed with 0.5 mg/mL of collagenase and dispase II (Sankyo, Tokyo, Japan), and 0.075% trypsin (Wako) in complement medium at 37°C for 20 min. Kidney single cells were finally obtained by passing the samples through a 25-μm filter to remove incompletely dissociated renal tissue. For the purification of podocytes, kidney single cells (1 × 10^6) were incubated with 2.5 μg of biotin anti-CD31 monoclonal antibody (Biolegend, San Diego, CA, USA) followed by an incubation with Streptavidin MicroBeads (Miltenyi Biotec, Bergisch Gladbach, Germany), and CD31-positive endothelial cells were then excluded using the octo-MACS system with MS columns (Miltenyi). The CD31-negative fractions passed through the magnetic columns were subsequently subjected to the second MACS separation using anti-nephrin antibody. The cells (this case) were labelled with 10 μg of biotin-conjugated antibody recognizing extracellular region of nephrin followed by another incubation with Streptavidin MicroBeads and separation using the octoMACS system. The positive and negative fractions of the second MACS separation were collected as nephrin-positive and nephrin-negative fractions, respectively.

All animal studies were approved by the Animal Institute Committee of Yokohama City University School of Medicine.

Confocal microscopy analysis

Cell suspensions of nephrin-positive and nephrin-negative fractions were attached to microscope slides using a Cytospin centrifuge (Sakura Finetek, Tokyo, Japan), then fixed with 2% paraformaldehyde for 30 min at 4°C, permeabilized with 0.3% Triton for 30 min at room temperature, and blocked with 2% BSA for another 30 min at room temperature. Slides were incubated with Alexa Fluor 488 anti-rabbit IgG (Life Technologies) and mouse anti-synaptopodin (Progen, Heidelberg, Germany) overnight at 4°C, washed in PBS, and incubated with Alexa Fluor 596 anti-mouse IgG (Life Technologies, Carlsbad, CA, USA) for 30 min at room temperature. Nuclear staining was performed with Hoechst 33342 (Life Technologies).

Mouse kidneys were snap-frozen, and cryosections of 5 μm in thickness on glass slides were prepared. After fixation with acetone for 5 min at 4°C and subsequent blocking with 2% BSA in PBS, sections were subjected to double staining with anti-nephrin antibody and anti-podoplanin (Biolegend, San Diego, CA, USA) or anti-CD31 (Biolegend), followed by an incubation with appropriate Alexa Fluor-conjugated secondary antibodies. Hoechst 33342 was added for nuclear staining.

Slides were observed with a FV300 confocal laser microscopy (Olympus, Tokyo, Japan) immediately after immunostaining.
Flow cytometry

Cells (1 × 10^5) collected from each fraction were blocked by incubation in fluorescence-activated cell sorting (FACS) buffer (0.1% sodium azide and 0.2% BSA/PBS) containing 10% normal goat and hamster serum, and 0.5 μg of CD16/CD32. After washing with FACS buffer, the cells were stained with the antibody against the extracellular part of nephrin and Alexa Fluor 488-conjugated anti-rabbit IgG antibody (Life Technologies) at 4°C for 30 min. After washing with FACS buffer, the cells were analysed using FACSCanto II (BD Biosciences).

Immunoblotting

Lysates of kidney single cells, and nephrin-positive and nephrin-negative fractions were prepared using RIPA buffer containing protease inhibitors (Thermo Fisher Scientific). HEK293 cells were maintained in Dulbecco's Modified Eagle's Medium (Life Technologies) supplemented with 10% FBS, penicillin and streptomycin. Expression vector pcDNA3.1 containing full length of mouse nephrin was kindly provided by Dr Holzman (University of Pennsylvania School of Medicine, PA, USA). Transfection was carried out with Lipofectamine 2000 (Life Technologies) according to the manufacturer’s protocol. After 24 h, cell lysates were prepared using RIPA buffer containing proteinase inhibitors. These cell lysates (2 μg) were resolved by SDS–PAGE, and the proteins were detected by immunoblotting using the ECL detection system (PerkinElmer, Waltham, MA, USA). To determine the expression level of nephrin, our antibody against intracellular region of nephrin was used. Anti-WT1 (F-6), anti-aqp3 (C-18) and secondary antibodies conjugated with horseradish peroxidase were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). β-Tubulin antibodies were purchased from Imagenex (San Diego, CA, USA).

Fig. 1. Immunoblot analysis of lysates of single cells obtained from mouse kidneys (lane 1). Lysates of HEK293 cells transfected with expression vector of mouse nephrin (lane 2) or mock vector (lane 3) were also applied. A PVDF strip was incubated with an antibody raised in rabbits against mouse nephrin intracytoplasmic region (nephrin (c)), or antibody against nephrin extracellular region (nephrin (e)). A strip was also incubated with an antibody pre-incubated with each recombinant antigen (+ Ag).

Fig. 2. Confocal microscopic analysis of mouse kidneys using an antibody against mouse nephrin intracytoplasmic region (A) or extracellular region (B). The sections were also stained with anti-podoplanin or anti-CD31 antibody. Nuclear staining was performed with Hoechst 33342.
Real-time PCR

cDNA was prepared using a Prime script RT reagent kit (Takara, Shiga, Japan), and PCR was then performed using SYBR premix Ex Taq II (Takara) with a Thermal Cycler Dice Real Time System (Takara). The cDNA concentrations were normalized to the cycle threshold value of β-actin (18–22 cycles) and subjected to real-time PCR using Rox dye as a reference.

Statistical analyses

Data are expressed as the mean±SEM. Statistical significance was determined using the Student's t-test. All statistical calculations were performed using KaleidaGraph (Synergy Software, Reading, PA, USA).

Results

Using lysates of kidney single cells and HEK293 cells transiently transfected with full-length mouse nephrin our antibodies raised against the intracellular (amino acids 1099–1177) and extracellular (amino acids 768–832) regions of nephrin specifically recognized the clear bands with molecular sizes consistent with nephrin as previously reported (Figure 1) [10,11]. The difference between molecular weight of the immunoblots using kidney single cells and HEK293 cells would be attributed to distinct patterns of posttranslational modification, especially N-glycosylation, of nephrin [11]. In contrast, no reactivity was found in the strips of the same lysates incubated with antibodies pre-incubated with recombinant antigens (Figure 1). The specific immunoreactivity of these antibodies against nephrin was further confirmed on immunofluorescence studies; our antibodies against nephrin clearly showed glomerular-specific staining with significant co-localization with podoplanin, another podocyte-specific marker (Figure 2). Furthermore, both anti-nephrin antibodies demonstrated clearly distinct expression patterns from CD31-positive endothelial cells (Figure 2). This antibody against the extracellular region...
of nephrin was utilized for positive selection of podocytes through the MACS system after endothelial cell depletion. The numbers of kidney single cells extracted from one mouse were $2.9 \pm 0.3 \times 10^7$, and $1.0 \times 10^7$ of these cells were used for each magnetic separation. The entire procedure for sorting nephrin-positive fractions took 4–5 h, and the fractions contained $15.1 \pm 1.9 \times 10^4$ per $10^7$ kidney single cells ($n = 10$). Confocal microscopy demonstrated that $\sim70–80\%$ of the positively sorted cells were double positive for synaptopodin and rabbit IgG, while no cells showed such dual positivity in the nephrin-negative fractions (Figure 3A). FACS analysis revealed that the proportion of rabbit IgG-positive cells was enriched gradually as the sorting step proceeds; $44.5 \pm 3.1\%$ in the kidney single cell fraction, $63.7 \pm 0.7\%$ in the CD31-negative fraction and $75.1 \pm 0.6\%$ in the nephrin-positive fraction; indicating absolute numbers of extracted podocytes with our MACS technique were $11.9 \pm 0.4 \times 10^4$ per $10^7$ kidney single cells ($n = 5$). In contrast, $40.0 \pm 0.5\%$ of nephrin-negative fraction showed positivity for anti-rabbit IgG ($n = 5$) (Figure 3B and C). Real-time PCR analysis showed a significantly higher expression of podocyte-specific molecules in the nephrin-positive fractions, including nephrin, podocin and WT1, compared with nephrin-negative fractions (Figure 4A). In contrast, the expression levels of aquaporin 1 and 3 (markers for the renal tubules and collecting ducts, respectively), and megsin (a mesangial marker) were significantly lower in the nephrin-positive fractions (Figure 4A). The amount of protein retrieved from the nephrin-positive fraction was $28.8 \pm 0.5 \mu g$ per $10^7$ kidney single cells ($n=6$), and immunoblot analysis further confirmed the enrichment of podocytes in the nephrin-positive fraction.

Fig. 4. mRNA and protein expression profiles of the separated fractions with anti-nephrin antibody. (A) Total RNA extracts of $8 \times 10^4$ nephrin-positive cells, nephrin-negative cells or CD31-positive cells were subjected to real-time PCR analysis of nephrin, podocin, WT1, aquaporin 1 (AQP1), aquaporin 3 (AQP3) and megsin (*$P<0.05$; **$P < 0.01$). (B) Immunoblot analysis of freshly isolated protein lysates from each fraction of three individual mice (no. 1–3).
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i.e. nephrin and WT1 were exclusively expressed, whereas aquaporin 3 was found only in the nephrin-negative fractions (Figure 4B).

Discussion

Since the identification of nephrin [12], a greater understanding of podocyte biology has been central to the issue of clarifying the mechanisms underlying glomerular injury. Various podocyte-specific molecules have now been identified [13–15], and many groups have reported that mutations or deletions of the genes encoding these proteins, either in humans or in genetically manipulated mice, result in severe proteinuria and premature death [12,15–17]. More recent studies have further revealed that several signalling cascades involving podocyte-specific molecules are critical for podocyte homeostasis [18–20], and then, the elucidation of the cellular events that occur during podocyte injury has become a primary focus in the field of podocytology.

Here, we describe a novel, rapid method using MACS using the anti-nephrin antibody to isolate these cells. Although the purity of the isolated podocytes is comparable to the conventionally established method for the primary cultures [21], it is particularly noteworthy that this novel method enables us to complete enrichment of podocytes and subject them to further analysis within 6 h after removal of a mouse kidney. Hence, our MACS technique is of great potential utility in examining podocyte biology in greater detail along with in vitro studies using established podocyte cell lines. Isolation of these cells from transgenic mouse models would also advance research in the field of glomerular biology.

Applying our MACS technique for isolating podocytes from mouse models of glomerular diseases could be challenging. Human nephrin has been reported to be downregulated or redistributed in the cytoplasm in nephritic syndromes, and similar expression manner was reported even in rodent models of minimal change disease [22–25]. However, some groups failed to observe such an expression pattern of nephrin [26,27], and upregulation of nephrin was even reported at early stages of glomerular dysfunction in anti-Thy1.1-induced glomerulonephritis and rodent models of diabetic nephropathy [28,29]. The expression pattern of nephrin in the proteinuric state is thus still controversial, and could be variable among the types of podocyte injury and dependent upon the time course of loss of glomerular filtration function. The purity of cells isolated with MACS technique depends on the molecules expressed on the surface of the target cells; therefore, the expression level and pattern of nephrin should be determined prior to utilizing our method to isolate podocytes from murine models of glomerulonephritis, or other surface markers such as nephl, neph2 or ZO-1 would be considered for labelling podocytes under proteinuric state. In this context, B7-1 might be a candidate molecule for podocyte isolation since its expression is induced only in the state of podocyte injury developed by several different mechanisms [30]. B7-1, however, is expressed on the surface of a variety of immune cells including B-cells, T-cells, dendritic cells and macrophages [31]; therefore, these inflammatory cells should be eliminated before podocyte isolation by utilizing B7-1 expression. It would be further interesting to identify a novel marker for podocytes only expressed in the state of glomerular injury for isolating them more specifically with MACS technique.

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Conflict of interest statement. None declared.

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Down-regulation of core 1 β1,3-galactosyltransferase and Cosmc by Th2 cytokine alters O-glycosylation of IgA1

Koshi Yamada1, Noriyoshi Kobayashi1, Tomomi Ikeda2, Yusuke Suzuki1, Toshinao Tsuge1, Satoshi Horikoshi1, Steven N. Emancipator3 and Yasuhiko Tomino1

1Division of Nephrology, Department of Internal Medicine, Juntendo University Faculty of Medicine, Tokyo, Japan, 2Division of Molecular and Biochemical Research, Juntendo University Graduate School of Medicine, Tokyo, Japan and 3Department of Pathology, Case Western Reserve University, Cleveland, OH, USA

Correspondence and offprint requests to: Yasuhiko Tomino; E-mail: yasu@juntendo.ac.jp

Abstract

Background. Patients with IgA nephropathy (IgAN) have an increased amount of abnormally O-glycosylated IgA1 in circulation, in glomerular deposits and produced by tissue cells in vitro. Although increased production of Th2 cytokines by peripheral blood lymphocytes and a functional abnormality of core 1 β1,3-galactosyltransferase (C1β3Gal-T) have been proposed as mechanisms underlying pathogenesis of IgAN, they are still obscure and are not connected.

Methods. To clarify the effect of T-cell cytokines, we analysed the mRNA levels of C1β3Gal-T and its molecular chaperone Cosmc, C1β3Gal-T activity and subsequent O-glycosylation of IgA1 in a human B-cell line stimulated with these cytokines. The surface IgA1-positive human B-cell line was cultured with recombinant human IFN-γ, IL-2, IL-4 or IL-5. The production and glycosylation of IgA1 were determined by sandwich ELISA and enzyme-linked lectin binding assay, respectively. The mRNA levels of C1β3Gal-T and Cosmc were quantitatively measured by real-time PCR. C1β3Gal-T activity was analysed using high-performance liquid chromatography.

Results. IgA1 production by IL-4-stimulated cells was significantly higher than controls or after IFN-γ or IL-5. The terminal glycosylation of secreted IgA1 was altered in response to IL-4. IL-4 stimulation significantly decreased the mRNA levels of both C1β3Gal-T and Cosmc and of C1β3Gal-T activity. IL-4 stimulation was clearly blocked by recombinant human IL-4 soluble receptor.

Conclusions. It appears that Th2 cytokine IL-4 may play a key role in controlling glycosylation of the IgA1 hinge region.

Keywords: IgA1 hinge region; T-cell cytokines; the surface IgA1-positive human B-cell line; Th2 response