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Quantitative analysis of markers of podocyte injury in the rat puromycin aminonucleoside nephropathy model

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ABSTRACT

Podocytes are an essential component of the renal glomerular filtration barrier, their injury playing an early and important role in progressive renal dysfunction. This makes quantification of podocyte marker immunoreactivity important for early detection of glomerular histopathological changes. Here we have specifically applied a state-of-the-art automated computational method of glomerulus recognition, which we have recently developed, to study quantitatively podocyte markers in a model with selective podocyte injury, namely the rat puromycin aminonucleoside (PAN) nephropathy model. We also retrospectively investigated mRNA expression levels of these markers in glomeruli which were isolated from the same formalin-fixed, paraffin-embedded kidney samples by laser microdissection. Among the examined podocyte markers, the immunopositive area and mRNA expression level of both podoplanin and synaptopodin were decreased in PAN glomeruli. The immunopositive area of podocin showed a slight decrease in PAN glomeruli, while its mRNA level showed no change. We have also identified a novel podocyte injury marker β-enolase, which was increased exclusively by podocytes in PAN glomeruli, similarly to another widely used marker, desmin. Thus, we have shown the specific application of a state-of-the-art computational method and retrospective mRNA expression analysis to quantitatively study the changes of various podocyte markers. The proposed methods will open new avenues for quantitative elucidation of renal glomerular histopathology.

1. Introduction

Nephropathy patients are increasing worldwide and becoming a global health problem. Interestingly, injury of cells called podocytes occurs at the early stage of glomerular damage (Wiggins, 2007). Podocytes are located on the outside of glomerular capillaries, forming a specialized cell-cell junction termed slit diaphragm and serve as a filtration barrier (Pavenstädt et al., 2003). Many proteins including the slit diaphragm complex proteins are exclusively expressed in podocytes and are presumed to play a crucial role in function and functional disturbances. Morphological changes of podocytes in glomerular damage comprise foot process effacement and eventual detachment from glomerular basement membranes. Along with morphological changes, podocyte-specific proteins are downregulated, impairing the integrity of the slit diaphragm. Thus, the decrease of the podocyte specific proteins has been used to mark podocyte injury (Breiteneder-Geleff et al., 1997; Kawachi et al., 2003; Yanagida-Asanuma et al., 2007). In addition to the decrease of the podocyte-specific proteins, damaged podocytes incur the upregulation of mesenchymal proteins such as desmin (Li et al., 2008; Liu, 2010). The increase of desmin immunostaining in glomerular podocytes has often been suggested as a podocyte injury marker (Floege et al., 1992; Hoshi et al., 2002; Zou et al., 2006; Herrmann et al., 2012; Sofue et al., 2012; Hirohashi et al., 2014).

Since podocyte injury occurs at the early stage of glomerular damage, quantification of podocyte injury markers is quite important to quantitatively evaluate early glomerular disease. Although the quantification of glomerular immunopositive area was reported in a few studies (Herrmann et al., 2012), these authors

Abbreviations: FFPE, formalin-fixed, paraffin-embedded; HOG, histogram of oriented gradients; PAN, puromycin aminonucleoside; SVM, support vector machine.

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manually traced each glomerulus border to segregate glomeruli from other kidney structures. Because this approach was inherently quite laborious and time consuming, they examined only a few glomeruli out of more than 100 in a kidney specimen. Considering the heterogeneity of glomerular injuries in renal cortex (Sofue et al., 2012; Hirohashi et al., 2014), the limited number of glomeruli ultimately subjected to analysis carries the risk of arbitrary selection bias.

To solve these problems, we have recently developed a novel method, applying the state-of-the-art computational method used in face- and car-recognition systems, to detect glomeruli (Hirohashi et al., 2014). Specifically, we have utilized the linear support vector machine (SVM) learning system combined with a histogram of oriented gradients (HOG) object detection algorithm to automatically recognize glomeruli and quantify glomerular immunopositive area. The automated, computational image analysis method has enabled automatic recognition and quantification of glomerular injury, eliminating the need to laboriously trace glomerulus borders by hand.

In this study, we have quantitatively studied the immunoreactivities of various podocyte markers in puromycin aminonucleoside (PAN) toxicity-induced nephropathy (Zou et al., 2006; Herrmann et al., 2012) with overt proteinuria and selective podocyte injury to establish whether this automated, rapid and objective technique will facilitate the study of podocyte pathophysiology. We also retrospectively examined mRNA expression levels of the podocyte markers in glomeruli which were isolated from the same formalin-fixed, paraffin-embedded (FFPE) kidney samples by laser microdissection. By these methods, we investigated whether changes of immunoreactivity and mRNA expression level correlated in the same sample of kidney tissue. In addition, we have found a novel podocyte injury marker β-enolase which was exclusively increased in PAN rat glomeruli. The increase of β-enolase in renal glomeruli could well be established as a novel podocyte injury marker for glomerular damage.

2. Materials and methods

2.1. Animals

Five-week-old male Sprague-Dawley rats were purchased from Charles River Laboratories Japan (Yokohama, Japan), and acclimatized for 1 week prior to study. The animals were housed under a 12-h light/12-h dark cycle and allowed free access to water and chow. The animals were injected with 100 mg/kg of PAN (Sigma-Aldrich, St. Louis, MO) or saline as vehicle, via a tail vein (4 animals each). On day 7, timed 24-h urine was collected using individual metabolic cages, whereafter the animals were sacrificed by exsanguination under sevoflurane anesthesia. All animal experiments were in accordance with the institutional guidelines, and approved in advance by the Committee of Animal Experiments in the Research Division of Mitsubishi Tanabe Pharma Corporation.

2.2. Urinary protein

Urinary protein levels were determined by Rat Urinary Protein Assay Kit (Chondrex, Redmond, WA), according to the manufacturer's instructions.

2.3. Immunohistochemistry

The kidneys removed from the euthanized animals were immediately fixed in 10% neutralized buffered formalin and embedded in paraffin for histological analysis. Immunohistochemistry was performed as described previously (Kakimoto et al., 2013; Kakimoto et al., 2014). Briefly, kidney paraffin sections were deparaffinized and then incubated overnight at 4 °C with anti-podoplanin mouse monoclonal antibody (AngioBio, Del Mar, CA), anti-synaptopodin mouse monoclonal antibody (Progen, Heidelberg, Germany), anti-podocin rabbit polyclonal antibody (Sigma-Aldrich), anti-desmin mouse monoclonal antibody (Dako, Glostrup, Denmark) and anti-β-enolase mouse monoclonal antibody (Santa Cruz Biotechnology, Santa Cruz, CA). Treatment with primary antibody was followed by incubation with horseradish peroxidase-conjugated secondary antibody (Nichirei, Tokyo, Japan). The sections were colorized with 3,3′-diaminobenzidine and counterstained with hematoxylin. The sections were photographed with a DP73 digital camera system (Olympus, Tokyo, Japan) and cellSens software (Olympus) equipped with a BX51 microscope (Olympus).

For immunofluorescence microscopy, kidney paraffin sections were deparaffinized and incubated overnight at 4 °C with anti-β-enolase mouse monoclonal antibody followed by incubation with alkaline phosphatase-conjugated secondary antibody (Nichirei) and colorization with Liquid Permanent Red (Dako). Thereafter, the sections were incubated overnight at 4 °C with anti-desmin mouse monoclonal antibody followed by incubation with Alexa 488-conjugated secondary antibody (Life Technologies, Carlsbad, CA). The sections were photographed with an AxioCam MRm digital camera system (Carl Zeiss, Oberkochen, Germany) and AxioVision software (Carl Zeiss) equipped with an Axio Imager A2 microscope (Carl Zeiss).

2.4. Image analysis

Whole slide digital images of the immunostained sections were obtained with Aperio Scan Scope XT (Leica Microsystems, Wetzlar, Germany). We utilized a novel computational method which we recently developed (Hirohashi et al., 2014) to automatically detect glomeruli and to quantify the glomerular immunopositive area. Briefly, a 200 × 200-pixeled detection window scanned whole slide image and converted sub-images into 512-dimensional HOG feature vectors. A linear SVM classified a sub-image as glomerulus if the SVM score was over the optimal threshold specified by training in advance. Next, boundaries of the detected glomeruli were automatically determined by using standard image processing including edge detection, smoothing, binarization, and morphology operation, followed by the quantification of immunopositive area. Mostly, more than 120 glomeruli were automatically recognized and analyzed for each rat. For podoplanin immunostaining of PAN rat kidneys, the numbers of automatically recognized glomeruli were decreased to 59–113 for each rat, because of a marked decrease in glomerular podoplanin immunoreactivity.

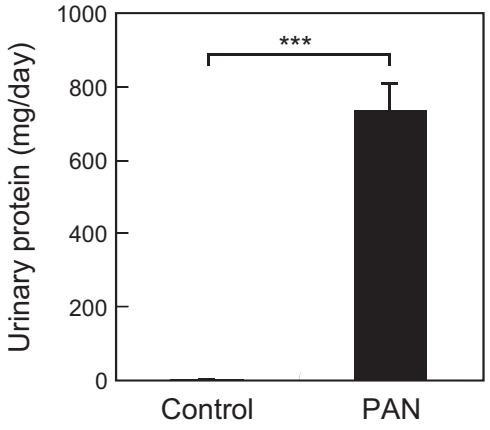

Fig. 1. Urinary protein levels of control and PAN rats. Statistically significant differences are indicated by *** ($p < 0.001$) (Student's t test).