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Semi-automated quantitative image analysis of podocyte desmin immunoreactivity as a sensitive marker for acute glomerular damage in the rat puromycin aminonucleoside nephrosis (PAN) model

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ABSTRACT

Glomerular visceral epithelial cells or podocytes are crucial for glomerular function and podocyte damage has been shown to be inevitably involved in glomerulopathies. Podocytes react to injury in a stereotypic manner. Accompanying morphologic changes is altered expression of intermediate filaments. Desmin is strongly upregulated in injured podocytes. Here we show, that semi-automated quantitative image analysis of desmin immunoreactivity in glomerula is a valid and sensitive marker for acute podocyte and thus glomerular damage in the puromycin aminonucleoside nephrosis (PAN) model in the rat with the potential of an efficacy marker in animal disease models as well as a toxicity marker for podocyte injury. Additionally, a panel of acknowledged urinary kidney biomarkers was evaluated for utility in the PAN model.

1. Introduction

Podocytes are postmitotic, highly differentiated epithelial cells with limited capacity to divide and therefore to be replaced if lost and serve various functions in glomerular physiology (Barisoni et al., 2007; Kim et al., 2001). They regulate glomerular permselectivity, give structural support for the glomerular capillary, cooperate with mesangial cells to resist the distensive force of intracapillary hydraulic pressure, they are involved in remodeling the glomerular basement membrane and endocytose filtered proteins (Ina et al., 2002; Kriz et al., 1994b; St John and Abrahamson, 2001; Tryggvason and Wartiovaara, 2001). Recent studies have shown that independent from the underlying cause, podocyte damage is inevitably involved in glomerular diseases, which in turn account for 90% of end-stage kidney disease (Barisoni et al., 2007; Wiggins, 2007).

From a pathogenetic point of view the podocyte can be targeted by a numerous amount of insults, e.g. toxic, genetic, immune, infectious, oxidant, metabolic or hemodynamic, which results in a broad spectrum of clinical syndromes including for example immune and inflammatory glomerulonephropathies, hypertensive nephropathy and diabetic glomerulonephropathy (Wiggins, 2007). From a morphologic point of view, podocyte damage seems to manifest in a fairly stereotypic manner (Kriz, 1997; Kriz et al., 1994a). Morphologic changes comprise cell hypertrophy, foot process effacement, pseudocyst formation, cytoplasmic overload with reabsorption droplets, detachment from the glomerular basement membrane and apoptosis (Durvasula and Shankland, 2006; Kriz, 1997; Shankland, 2006; Wiggins, 2007).

The outcome of podocyte damage depends on the occurrence of podocyte depletion. If podocytes are not depleted, the glomerulus can recover to normal structure and function (Wiggins, 2007). If significant podocyte depletion occurs this will lead to progressive glomerulosclerosis (Kriz, 1997; Kriz et al., 1998).

Going along with morphologic changes are changes in the phenotype of injured podocytes, which have been referred to as epithelial-to-mesenchymal transition. Podocytes derive from metanephric mesenchyme through mesenchymal-to-epithelial transdifferentiation and have been shown to regain the potential to reexpress mesenchymal proteins (desmin, collagen 1 and fibronectin) (Li et al., 2008) under the influence of transforming growth factor-beta, which is upregulated in diseased kidneys (Liu, 2006). On the other hand important components of the slit diaphragm like nephrin are suppressed which is likely to impair the integrity of the slit diaphragm and might thus be involved in the morphologic outcome of podocyte injury (Li et al., 2008).

Desmin has often been suggested a podocyte injury marker and apart from the PAN model its upregulation has been described in various glomerular disease models like Masugi nephritis, Heymann nephritis, diabetic nephropathy and age-related glomerulosclerosis (Floege et al., 1992; Floege et al., 1997; Hoshi et al., 2002; Yaoita et al., 1990). In the PAN model, differential desmin staining has been described in control and treated rats. In control rats desmin is mainly located within mesangial cells and vascular smooth muscle cells with very weak staining in podocytes, while in PAN enhanced

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desmin staining is observed in podocytes (Zou et al., 2006). The time course of expression, which was also established in the PAN model revealed that desmin mRNA did not increase before day 4 and rose to 9.1-fold at day 10 (Zou et al., 2007).

The PAN model is a well-established and recognized model for podocyte damage (Fishman and Karnovsky, 1985; Lannigan et al., 1962; Ryan and Karnovsky, 1975). The podocyte injury in the PAN model is thought to be caused by oxygen free radicals (Diamond et al., 1986), which are generated from hypoxanthine, an intermediate metabolite of PA (Nagasawa et al., 1967). It is assumed, that hypoxanthin serves as a substrate for superoxide anion production via the xanthine oxidase system (Diamond et al., 1986). Early lesions in PAN are loss of foot processes and appearance of focal defects in the epithelial covering of the glomerular basement membrane going along with early massive proteinuria (Ryan and Karnovsky, 1975) and inevitably lead to glomerulosclerosis (Kim et al., 2001).

Here we show that quantitative image analysis of immunohistochemical desmin staining in renal glomerula is a valid and sensitive marker for acute podocyte damage in the rat and could well be established as an efficacy marker in animal models of acute glomerular damage as well as a toxicity marker to demonstrate dose-dependent glomerular damage by substances causing podocyte injury. Additionally, we present the first evaluation of a panel of acknowledged urinary kidney biomarkers for the PAN model.

2. Experimental design

Male Sprague-Dawley rats (75–100 g, 3–4 weeks old) were delivered to the F. Hoffmann-La Roche Basel animal facility from Charles River Laboratories (Germany) and were housed individually at approximately 21 °C temperature, 55–65% relative humidity, and a 12:12 h light:dark cycle. After a 5-day acclimation period, they were block randomized according to body weight, in groups of 6–8 rats. Animals were given water and chow ad libitum at all times. Body weight was measured before treatment start and daily throughout the treatment. On day −1 of the study, the animals were placed in individual metabolic cages to determine baseline 24-h albumin/creatinine excretion in urine. On day 1, rats were injected intraperitoneally with 12.5, 25, 50, 75, 100 or 125 mg/kg of PAN or vehicle (0.9% NaCl). Timed 24-h urine was collected from days 4 to 7, at which time animals were sacrificed and kidneys were dissected for analysis. Blood withdrawal was performed after sacrifice for plasma parameter measurements.

Experiments were conducted under authorization from the Swiss Federal Veterinary Office and the Association for Assessment and Accreditation of Laboratory Animal Care International.

3. Histology and immunohistochemistry

One half kidney was fixed in 10% neutral-buffered formalin for 24 h, embedded in paraffin and cut longitudinally at 4 μm. Specimen were stained with H&E and PAS for histopathologic evaluation. On consecutive slides desmin immunohistochemistry was performed on the Ventana Nexes® PA II 0002 immunostainer with anti-desmin rabbit monoclonal antibody clone Y66 (Millipore, 04-585) as primary antibody in a standard protocol using the Ventana iView™ detection system kit (05266157001). Vascular smooth muscle within the kidney served as an internal positive control.

4. Image analysis

Image analysis was performed with a ruleset recognizing the pixel area of desmin staining in glomeruli using Definiens Developer™ XD1.1 software. Images at obj ×40 were taken from specimen of all animals dosed 25, 50 and 75 mg/kg and 5 randomely chosen glomeruli were analyzed per animal. The average pixel area of desmin per glomerulum was calculated for each animal.

5. Urine analysis

Urine albumine and creatinine were measured with a rat albumin EIA from ALPCO and a Roche Diagnostics kit in a Hitashi analyzer respectively.

Urine samples were sent to Rules Based Medicine (Austin, Texas, USA) to perform multiplexed ELISA on a panel of generally accepted markers of kidney injury (β2-microglobulin, clusterin, cystatin-c, KIM-1, NGAL, and VEGF).

Table 1
Summary of histopathologic findings in control animals and animals dosed 25, 50 or 75 mg/kg puromycin aminonucleoside; severity is graded as follows: 0 (not present), 1 (minimal), 2 (slight), 3 (moderate), 4 (marked), 5 (severe).

	1	2	3	4	5	6
0 mg/kg						
Glomerular changes						
Glomerular hypertrophy	0	0	0	0	0	0
Mitoses/apoptoses increased	0	0	0	0	0	0
Mesangial distension	0	0	0	0	0	0
Podocytes: hyaline droplets	0	0	0	0	0	0
Tubular changes						
Tubular casts	0	0	0	0	0	0
Proximal tubular necrosis	0	0	0	0	0	0
Proximal tubular regeneration	0	0	1	1	0	0
Proximal tubules: hyaline droplets	0	0	0	0	0	0
	7	8	9	10	11	12
25 mg/kg						
Glomerular changes						
Glomerular hypertrophy	1	0	0	0	0	0
Mitoses/apoptoses increased	0	0	0	0	0	0
Mesangial distension	0	0	0	0	0	0
Podocytes: hyaline droplets	0	0	0	0	0	0
Tubular changes						
Tubular casts	0	0	0	0	0	0
Proximal tubular necrosis	0	0	0	0	0	0
Proximal tubular regeneration	0	1	0	1	0	0
Proximal tubules: hyaline droplets	0	0	0	0	0	0
	13	14	15	16	17	18
50 mg/kg						
Glomerular changes						
Glomerular hypertrophy	1	1	1	1	1	1
Mitoses/apoptoses increased	1	1	0	1	1	1
Mesangial distension	1	1	0	1	1	1
Podocytes: hyaline droplets	1	1	1	1	1	1
Tubular changes						
Tubular casts	0	0	0	0	0	0
Proximal tubular necrosis	0	0	0	0	0	1
Proximal tubular regeneration	1	0	1	0	0	1
Proximal tubules: hyaline droplets	0	0	0	0	0	0
	19	20	21	22	23	24
75 mg/kg						
Glomerular changes						
Glomerular hypertrophy	1	1	2	0	2	2
Mitoses/apoptoses increased	1	2	2	1	2	2
Mesangial distension	2	2	2	1	3	3
Podocytes: hyaline droplets	3	2	2	4	4	3
Tubular changes						
Tubular casts	2	3	5	0	3	3
Proximal tubular necrosis	1	1	2	0	0	0
Proximal tubular regeneration	1	2	2	0	0	3
Proximal tubules: hyaline droplets	0	1	4	0	2	3