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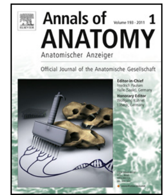

Design-based stereological methods for estimating numbers of glomerular podocytes

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SUMMARY

The podocyte depletion hypothesis has emerged as a unifying concept in glomerular pathology. According to this hypothesis podocyte depletion may be absolute (decrease in number of healthy mature podocytes), relative (fewer podocytes per unit of glomerular volume) or involve alterations to the specialized podocyte architecture (such as foot process effacement). To study and understand podocyte depletion it is important to be able to accurately and precisely count these cells. Here we present new design-based stereological methods for estimating podocyte number in individual glomeruli of known volume, and in average glomeruli. Both methods involve serial histological sectioning, triple label immunohistochemistry, laser confocal microscopy and cell counting with the optical disector/fractionator.

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1. Introduction

Podocytes are exclusively located within renal glomeruli (Fig. 1A and B) and together with glomerular endothelial cells and the glomerular basement membrane form the glomerular filtration barrier. Alterations to the integrity of podocytes and the filtration barrier are involved in the development and progression of a range of renal pathologies (Kriz and Endlich, 2012).

Podocytes have a highly specialized structure, consisting of a cell body (containing the nucleus, mitochondria, Golgi apparatus, rough endoplasmic reticulum and lysosomes) from which emanate large cytoplasmic processes (Fig. 1C). Foot processes (pedicels) in turn emanate from the large cytoplasmic processes and are attached to the basement membrane. Filtration slits (Fig. 1D) are located between adjacent foot processes and feature a slit diaphragm which regulates the flow of water and small molecules across the filtration barrier. In addition to their key roles as components of the glomerular filtration barrier, podocytes synthesize and secrete a range of growth factors as well as components of the glomerular basement membrane (Jefferson et al., 2011).

It has been known for many years that podocyte dysfunction is associated with a large number of primary and secondary glomerular pathologies including minimal change disease, focal and segmental glomerulosclerosis (FSGS), diabetic nephropathy, collapsing glomerulopathy, diffuse mesangial sclerosis, congenital nephrotic syndrome of the Finnish type, Alport's syndrome and obesity-related glomerulopathy (Wiggins, 2007). Despite the differences in etiology, glomerular pathology and clinical severity of these conditions, the podocyte depletion hypothesis has emerged in recent years as a unifying concept in glomerular pathology (Wiggins, 2007; Wharram et al., 2005; Fukuda et al., 2012; Kriz et al., 1996, 1998; Kriz, 1997, 2002). In brief, this hypothesis proposes that podocyte depletion, whether it be absolute, relative or involving phenotypic change, renders glomeruli susceptible to subsequent pathological change. Absolute podocyte depletion involves a loss of podocytes, via apoptosis, necrosis or detachment from the basement membrane (for a comprehensive review see (Tharaux and Huber, 2012)). Relative podocyte depletion occurs when a finite number of podocytes is required to cover an expanded glomerular filtration surface area or maintain a hypertrophied glomerular volume. Podocyte phenotypic changes include foot process effacement and cellular hypertrophy. The podocyte depletion hypothesis is predicated on the understanding that podocytes have little or no capacity for proliferation and thereby replacement. However, recent evidence suggests that some degree of podocyte

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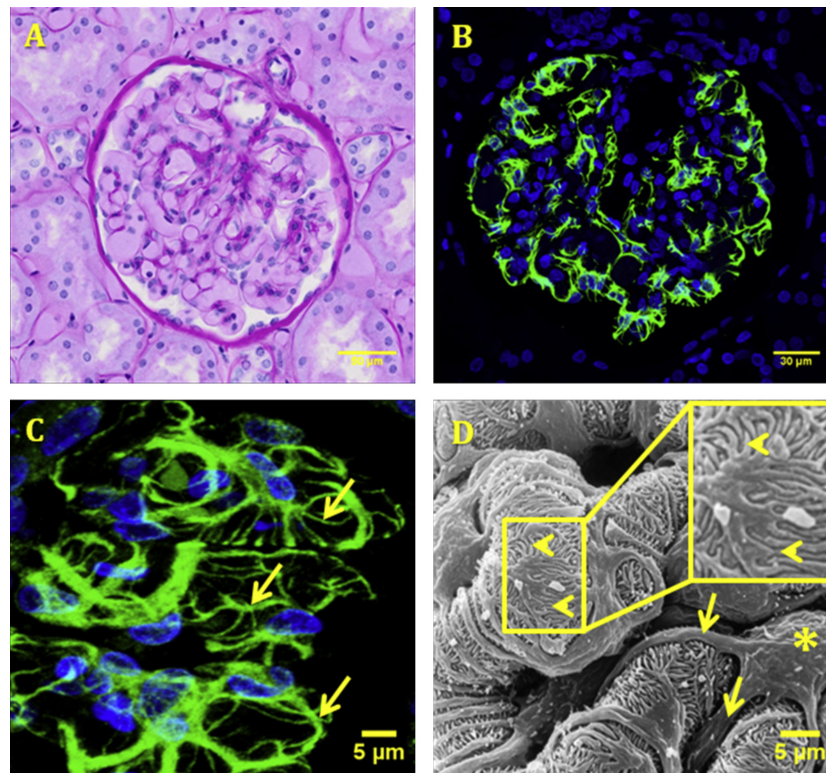

Fig. 1. Identifying glomerular visceral epithelial cells (podocytes) in the glomerulus. (A) Photomicrograph of a human glomerular profile in a PAS-stained glycolmethacrylate section (section thickness 10 µm). Scale bar = 50 µm; (B) confocal microscope image of an immunostained human glomerular profile showing podocyte cytoplasm (green – antibody against Wilms' Tumor 1-WT1). Nuclei are stained blue with DAPI. Scale bar = 30 µm; (C) 3D-reconstruction (Imaris, Bitplane, USA) based on confocal microscopic optical sections (1 µm apart) from a human glomerular profile showing podocyte cytoplasmic projections (arrows). Nuclei are stained blue with DAPI. Scale bar = 5 µm; and (D) scanning electron micrograph showing podocytes in a rat glomerulus. Podocyte cell bodies can be seen (*) together with major cytoplasmic processes (arrows) and interdigitating foot processes (arrowheads); inset figure clearly shows the interdigitating foot processes. The black lines between adjacent foot processes indicate filtration slits.

replacement may be possible via the proliferation and differentiation of a subpopulation of parietal epithelial cells (PECs) located close to the urinary pole (Sagrinati et al., 2006; Ronconi et al., 2009; Appel et al., 2009).

To study and understand podocyte depletion it is important to be able to accurately and precisely count these cells. Here we present new design-based stereological methods for estimating podocyte number in individual glomeruli of known volume, and in average glomeruli. Before describing these methods, we briefly discuss the methods currently available for estimating podocyte number.

2. Current methods for quantifying podocyte number

A range of methods are currently employed to estimate podocyte number. Perhaps the most commonly used method involves the counting and reporting of numbers of podocyte nuclear profiles (the bits and pieces of nuclei seen in histological sections) per glomerular cross-section (N_{gcs}) or per unit area of glomerular section (N_A). In both cases, it is important to note that it is not podocytes that are being counted but rather podocyte nuclear profiles. While these two approaches may appear valid, it turns out that the number of podocyte nuclear profiles seen in a section is not only related to the number of podocyte nuclei (and assuming one nucleus per podocyte, the number of podocytes) present, but also to podocyte nuclear shape and size, and section thickness. Moreover, neither method provides an estimate of the total number of podocytes in a glomerulus.

To overcome the problems above, a range of model-based stereological methods were developed in the twentieth century (Floderus and Flex, 1944; Wicksell, 1925; Abercrombie, 1946; Dehoff and Rhines, 1961; Weibel and Gomez, 1962). These methods provide estimates of podocyte nuclear numerical density (and thereby podocyte numerical density) in glomeruli (N_V, number of podocytes per unit volume of glomerulus). When N_V is multiplied by glomerular volume we obtain an estimate of the total number of podocytes in a glomerulus ($N_{pod,glom}$). However, these methods are designated "model-based" because they require knowledge of the geometry (mean caliper diameter, size, size distribution, shape) of the podocyte nucleus. Generally, values for these geometric parameters are assumed rather than measured, and therefore to the extent that these geometric assumptions are incorrect, the resultant estimates of podocyte number are biased.

In 1984, the publication of the disector method by Sterio (1984) revolutionized stereology because, for the first time, knowledge of particle (a 3-dimensional object such as a podocyte nucleus) geometry was not required to estimate number. The disector samples particles such as podocyte nuclei with equal opportunity, regardless of their size, size distribution, orientation or shape. The first iteration of the disector required the comparison of features in two physical sections – the physical disector. This method has been used by several groups to successfully count podocytes in rat (Bai and Basgen, 2011; Bertram et al., 1992) and human glomeruli (White et al., 2002; White and Bilous, 2004) at both the light and electron microscopic levels. In 1986, Gundersen described the optical disector, with which optical sections through tissue are compared and particles of interest then counted using the disector principle.

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