Podocyte Number in the Maturing Rat Kidney

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Key Words

Fractionator/disector • Glomerular volume • Podocyte number • Rat • Stereology

Abstract

Background/Aims: The podocyte is an important cell for maintaining the normal structure and function of the glomerulus. In recent years much attention has been given to the number of podocytes in glomeruli. During this time there has been a debate as to whether podocytes can divide after the capillary-loop stage of development. The aim of this study was to use an unbiased counting method to determine if podocyte number increases after the capillary-loop stage of development. **Methods:** The fractionator/disector method was used to count podocytes in glomeruli from rats aged 1 day, 5, 12, and 24 weeks. Glomerular volume was also measured with the unbiased Cavalieri principle and used to calculate the density of podocytes per glomerulus. **Results:** The number of podocytes did not increase from the capillary-loop stage of glomerular development to 24 weeks of age. Glomerular volume increased 3.6-fold during this time, which resulted in a decrease of podocyte density as the rats aged. **Conclusion:** The study documents that the number of podocytes is stable after the capillary-loop stage of glomerular development. The data does not confirm but adds evi-

dence that podocytes do not divide from the capillary-loop stage of glomerular development to 24 weeks of age in the normal rat.

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Introduction

The podocyte of the kidney plays a crucial role in both the structure and function of the normal glomerulus [1, 2]. Morphological change in the podocyte is one of the primary indications of glomerular dysfunction, with widening of foot processes and with proteinuria being an early sign of glomerular disease [3]. With advancing glomerular disease podocytes may detach from the glomerular basement membrane [4] and they have been found in urine of patients with different glomerular diseases [5]. It has been reported that podocytes do not divide after the capillary-loop stage of glomerular development [6], but other studies describe podocyte hyperplasia during glomerular growth [7] or in some glomerular diseases [8, 9]. If podocytes are found in the urine and if it is not possible to produce replacement podocytes, there should be a decrease of podocytes in patients with nephropathies. Pagtalunan et al. [10] first reported a decrease in podocyte number in Pima Indians with type II diabetes and

proteinuria. Since that initial report, there have been several other studies reporting a decrease in podocyte number in diabetes [11, 12] and other renal diseases [13, 14]. There have also been numerous studies reporting decreased podocyte numbers in rat [15, 16] and mouse [17] models of renal diseases.

Most of these studies used biased counting methods, which may have contributed to the controversy of whether podocytes can divide after the capillary-loop stage of development. To help clarify this important issue we designed a study to use the fractionator/disector, an unbiased sampling and counting technique [18, 19], to count podocyte number in the maturing rat glomerulus.

Materials and Methods

Tissue Preparation

Female Sprague-Dawley rats were kept in the Animal Center of Southern Medical University, Guangzhou, China according to the policy of the Committee for Animal Usage. At ages 1 day, 5, 12 and 24 weeks, 6 rats were anesthetized and their left kidney removed. From the cortex several 1-mm cubes were cut, fixed in 2.5% glutaraldehyde and embedded in Poly/Bed® 812 resin (Poly-science, Inc.). Using an ultramicrotome with a diamond knife, 300 sequential 1-μm thick sections were cut and pairs of adjacent sections were saved at 20-μm intervals from each of the rats.

Imaging

A standard microscope with a ×100 oil lens (NA = 1.25) and digital camera were used to obtain images. Photoshop® CS2 software (Adobe Systems, Inc.) was used to observe the images and count the cells.

Glomeruli were selected for imaging by observing the first section from the first saved section pair. All glomeruli present in that section were not eligible for analysis since an unknown number of podocytes were not available for counting. Each subsequent section pair was then observed and when a new glomerulus appeared it was imaged in the first and all subsequent section pairs that contain that glomerulus. For glomerular profiles containing the juxtaglomerular apparatus the limit of the glomerulus was defined by drawing a line between the 2 points on either side of the juxtaglomerular region where Bowman's Capsule reflects into the glomerulus. For each rat the first 5 complete glomeruli to appear were imaged and used for podocyte counting.

Podocyte Number

The cell boundaries of podocytes are not always resolved in the light microscope so podocyte nuclei were used as surrogates for the podocytes and were counted with the assumption there was 1 and only 1 nucleus per podocyte.

The fractionator/disector method was used to estimate the number of podocytes per glomerulus [19]. The disector is an unbiased sampling tool that consists of a pair of sections, the sampling section and the look-up section. One identifies and counts profiles of nuclei that are in the sampling section but not present in the look-up section (fig. 1). In order to determine the optimal

distance between sampling section and look-up section 5 serial sections were numbered 0, 1, 2, 3, and 4. Section 0 was observed with each of the other sections to determine when a nuclear profile in section 0 could always be determined to have come from the same nucleus as a profile in section 1, 2, 3, or 4. Only when section 0 was paired with section 1 was there no doubt as to whether profiles in the 2 sections came from the same nucleus (fig. 2). Thus the distance between the sampling and look-up sections was fixed at 1 μm.

The fractionator consists of obtaining an unbiased sample of a known fraction of sections from throughout the glomerulus. Thus if section pairs were collected every 20 μm from throughout the glomerulus, one twentieth of the glomerulus would be sampled.

The number of podocytes in a glomerulus was calculated using the equation,

$$N_{\text{podocyte}} = 20 \times \Sigma Q^- \quad (1)$$

where N_{podocyte} is the number of podocytes in the glomerulus, 20 is the reciprocal of the fraction of the glomerulus sampled, and ΣQ^- is the sum over all disector pairs of profiles from nuclei seen in the sampling section but not present in the look-up section (fig. 1). For the 1-day-old rats only glomeruli at the capillary-loop stage of development were used for counting.

Glomerular Volume

The Cavalieri principle was used to estimate glomerular volume [20]. Briefly, the 'layer' tool of Adobe Photoshop was used to randomly superimpose a point counting grid over each of the look-up sections from a glomerulus (fig. 3) and the number of points hitting the glomerular tuft profile was counted. For the definition of a 'point' consult Howard and Reeds' stereology text [21]. Glomerular volume was calculated using the equation

$$\text{Glomerular volume} = 20 \times [a(p)/3,050]^2 \times \Sigma P \mu\text{m}^3 \quad (2)$$

where 20 was the distance in micrometers between look-up sections, $a(p)$ was the area in micrometers square represented by 1 grid point, 3,050 was the magnification of the images, and ΣP was the total number of grid points hitting glomerular tuft from all the profiles from a glomerulus.

Numerical Density of Podocytes per Glomerulus

The density of podocyte per glomerulus [$N_v(\text{Podo}/\text{Glo})$] was calculated using the equation,

$$N_v(\text{Podo}/\text{Glo}) = N_{\text{podocyte}}/\text{glomerular volume nuclei}/\mu\text{m}^3 \quad (3)$$

Mesangial + Endothelial Cell Number and Density

As a control for the counting method we also counted the number of mesangial and endothelial cells using the fractionator/disector principle by substituting the number of Q^- counts for these cells in equation 1. The density of mesangial + endothelial cells was calculated by dividing the number of cells by glomerular volume, as in equation 3.

Statistics

ANOVA was used to determine if the number of cells, glomerular volume, and cell density at different times was statistically different. Data is given as mean $\pm$ SD (CE) where CE is the coefficient of error among glomeruli within a rat. CE is a measure of the variability and measuring error within an animal.

Fig. 1. A disector pair demonstrating profiles from the same podocyte nuclei in both the sampling section and the look-up section (red stars). Because profiles from the same nucleus are seen in both sections the nuclei are not counted. Profiles from 2 podocyte nuclei are seen in the sampling section but not in the look-up section (1 and 2) and thus 2 Q^- are counted in this disector pair.

Fig. 2. Images used to determine the optimal distance between the sampling section and the look-up section of a disector pair. All nuclear profiles seen in section +1 (1 μm from section 0) are easily identified as coming from the same nucleus as seen in section

0. Some profiles as far as 4 μm away (section +4) are easily identified as coming from the same nucleus (↓). But in section +4 there is a question if a profile (↑↑?) comes from the same nucleus as seen in section 0 (↑↑).

Fig. 3. Counting grid superimposed over glomerular profile at one sampling level. Nine grid points (+) were counted hitting the glomerular tuft and used to calculate glomerular volume.

Fig. 4. Individual rat data for the 5 measured parameters at each of the 4 time periods.

Results

Podocyte Number

The average number of disector pairs used per glomerulus for 1-day, 5-week, 12-week, and 24-week animals was 3.7, 4.0, 5.3, and 6.2, respectively, reflecting the increasing size of the glomeruli as they aged. The average number of Q⁻ counted per rat for each time period was 72, 63, 67, and 68. ANOVA analysis showed there was no change in podocyte number from the 1-day-old capillary-loop glomeruli to the glomeruli in the 24-week-old animals, $p = 0.60$. The number of podocytes per glomerulus at the 4 time periods was 144 ± 20 (0.12) [mean $\pm$ SD (CE)], 126 ± 24 (0.10), 134 ± 30 (0.15), and 137 ± 17 (0.15).

Glomerular Volume

To determine the mean glomerular volume an average of 170, 203, 419, and 621 grid points hit the glomeruli of each rat at the 4 respective time periods. The average glomerular volume at the 4 time periods was $182 \pm 51 \times 10^3 \mu\text{m}^3$, $218 \pm 52 \times 10^3 \mu\text{m}^3$, $449 \pm 104 \times 10^3 \mu\text{m}^3$, and $655 \pm 126 \times 10^3 \mu\text{m}^3$. ANOVA analysis showed a highly significant increase of glomerular volume during this time, $p < 0.001$.

Podocyte Density per Glomerulus

The density of podocytes per glomerulus decreased as the glomeruli aged, $p < 0.001$. The average density at the 4 time periods was $86 \pm 19 \times 10^{-5}/\mu\text{m}^3$, $63 \pm 11 \times 10^{-5}/\mu\text{m}^3$, $30 \pm 5 \times 10^{-5}/\mu\text{m}^3$, and $21 \pm 3 \times 10^{-5}/\mu\text{m}^3$, respectively.

Mesangial + Endothelial Cell Number

To estimate the mean number of mesangial plus endothelial cells an average of 69, 109, 167, and 210 Q⁻ were counted per rat for the 4 time periods. The number of mesangial plus endothelial cells increased with age, $p < 0.001$. The average number of these cells was 138 ± 39 , 215 ± 79 , 333 ± 86 , and 404 ± 82 for the four time periods.

Mesangial + Endothelial Cell Density per Glomerulus

There was a trend for the density of mesangial plus endothelial cells to increase in the younger rats but the density decreased in the older rats and thus there was no significant difference in density during the time studied. The average density for each of the 4 time periods was $77 \pm 13 \times 10^{-5}/\mu\text{m}^3$, $100 \pm 19 \times 10^{-5}/\mu\text{m}^3$, $76 \pm 9 \times 10^{-5}/\mu\text{m}^3$, $66 \pm 15 \times 10^{-5}/\mu\text{m}^3$, respectively.

The data for these 5 structural variables are shown in figure 4.

Discussion

In this study we used the fractionator/disector principle, an unbiased sampling and counting method to document the number of podocytes in rats from the capillary-loop stage of glomerular development to 24 weeks of age. Our study confirms that the number of podocytes per glomerulus in the normal rat is stable after the capillary-loop stage of development concomitant with a 3.6-fold increase in glomerular volume. The study also shows that as glomerular volume increases the number of mesangial plus endothelial cells increases 3-fold.

Our study does not confirm that podocytes cannot proliferate, only that the number of podocytes is stable during the time of glomerular maturation. It is possible that podocytes divide at the same rate they disappear, resulting in a stable number. Another possibility is the relatively recent idea that parietal epithelial cells migrate into the glomerular tuft and replenish disappearing podocytes [22].

The boundaries of glomerular cells are not resolvable by light microscopy, thus, we used the nucleus as the unambiguous marker for a cell. Nuclei are 3-dimensional particles, but we observed them in thin sections, obtained 2-dimensional images of the section, and then performed the cell counting on the 2-dimensional images. Wicksell pointed out more than 80 years ago that one cannot know the number of particles in 3-dimensional space by counting the number of particle profiles seen in a 2-dimensional sample from the 3-dimensional space [23]. Since Wicksell's paper there have been several suggestions for overcoming the Wicksell problem. Abercrombie assumed the particles to be counted were spheres and used the relationship between the particle diameters and the section thickness to create a correction factor [24]. Weibel and Gomez developed a method using a shape factor to try to overcome the Wicksell problem [25]. These and other methods use guesses as to the factor appropriate for the particular particles being counted. If the chosen shape factor is not the correct factor for the particle a biased estimate of number will result. If the particle shape or size changes under experimental conditions, comparison of the relative changes in particle number would not be valid. In 1984, Stereo introduced the disector principle for counting the numerical density of particles in 3-dimensional space using 2-dimensional samples [26]. The disector overcomes the Wicksell problem by using two 2-dimensional images a known distance apart, thus creating a 3-dimensional sampling space. Shortly after the introduction of the disector, Gundersen introduced the fractionator concept where a known fraction of sections through an object is used with

the disector to count the number of particles (nuclei) in the fraction. The number of particles counted is multiplied by the reciprocal of the fraction to obtain the total number of particles in the entire object [18]. When the fractionator principle is combined with the disector principle you have an unbiased method for counting the number of particles in 3-dimensional space using sections with no assumptions regarding shape, size, or orientation of the particles.

In an earlier study Olivetti et al. [7] counted podocytes in rats approximately 2 weeks and 7 weeks old and found a 50% increase in podocyte number over this time. The ages of rats in our study bracket the ages in that study but the studies reach different conclusions. We believe a reason for these results are the different counting methods. The Olivetti study used a single section to select nuclei to be counted. According to Wicksell [23] this would bias the count if the nuclei were of different size in the 2 groups. The volume of the nuclei were 58% larger in their 'adult' rats compared to their 'young' rats and thus their method should overestimate podocyte numbers in 'adult' compared to 'young' rats. The need for a compression correction and the critical measurement of section thickness may also add error to the Olivetti estimates. The fractionator/disector counts the number of nuclei independent of the size of the nuclei, section thickness, and sectioning compression.

When using the fractionator principle the larger the sample fraction the more precise the estimate of number. In fact, if a sampling fraction of 100% (all the sections through the glomerulus) is used there is no sampling error [27]. This of course takes much time and is not necessary. We chose a sample fraction of 1/20 as a reasonable amount of work. This resulted in an average coefficient of error of 0.13 which is equal to about 60% of the overall variation among the animals.

The disector method described here is known as the physical disector because the sampling and look-up sections are physically cut. An alternative method, the optical disector, uses thick sections ($>15\text{ }\mu\text{m}$) and an objective lens with a very high numerical aperture. The optical disector may be more efficient than the physical disector in laboratories properly equipped for using that method.

The time needed to obtain an unbiased estimate of cell number using the 2-section disector method is greater than the time necessary to obtain biased estimates using 1-section methods. However, the time needed to count cell number using either a biased or unbiased method is a small fraction of the overall time needed to carry out an entire experiment from the raising and treatment of the animals to processing and analyzing of samples. We be-

lieve using the disector counting method is well worth the additional fraction of overall experimental time to obtain an unbiased estimate of the true podocyte number.

The Cavalieri principle can be used to measure the volume of objects using sections a known distance apart and from throughout the object [20]. Thus, we were able to obtain precise estimates of individual glomerular volumes using the look-up section at each of the sampling levels from throughout each glomerulus. There was a large variation in volume in the capillary-loop stage glomeruli, $CV = 0.28$. This variation decreased somewhat with age but was still quite high at 12 weeks of age, $CV = 0.19$. This variation in glomerular volume may account for the variation in cell number among glomeruli of the same age. Also, glomeruli at the cortical-medullary border are often larger than glomeruli near the kidney surface. We did not differentiate among these various populations of glomeruli, which may have contributed to the variability in glomeru-

lar volume and podocyte number in this study. A more stable estimate of podocyte number may be the total number of podocytes per kidney, which possibly could result in a better correlation with a measure of kidney function.

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Disclosure Statement

The authors have no conflicts of interest to disclose.

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