Progressive Glomerular Injury in the MWF Rat Is Predicted by Inborn Nephron Deficit

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Abstract. It has been suggested that a reduced number of nephrons may predispose to systemic hypertension and glomerular injury. Compensatory hemodynamic changes, due to a low number of glomeruli, might be responsible for glomerular functional and structural changes. It is difficult to evaluate this hypothesis in humans because of limitations in estimating the number of nephrons in the living kidney. The aim of the present study was to estimate nephron number, single glomerular hemodynamics, and glomerular volume in male and female MWF rats, a strain that spontaneously develops systemic hypertension, proteinuria, and glomerulosclerosis. Male and female MWF rats were used as controls. At 12 to 14 wk of age, male MWF rats developed proteinuria, whereas female MWF and Wistar rats showed normal urinary protein excretion rates. Glomerular number was significantly reduced in male and female MWF rats (13,690 ± 1,489 and 12,855 ± 1,781 gl/kidney, respectively) compared with Wistar rats (26,955 ± 2,171 and 27,166 ± 1,754 gl/kidney, respectively). The mean number of nephrons per unit of body weight was also lower in MWF males (88 ± 10) compared with MWF females (139 ± 20) and compared with male and female Wistar animals (142 ± 14 and 221 ± 22 gl/g body wt). Whole-kidney hemodynamic parameters and the number of nephrons were used to calculate single-nephron filtration rate and plasma flow. Both measures were markedly elevated in male MWF rats relative to values obtained in the other three groups. Similarly, glomerular volume was significantly greater in MWF males than in other animals. These results suggest that an inborn deficit of nephrons may be responsible for spontaneous development of later-in-life hypertension and renal dysfunction. The data also indicate the need to investigate the role of this potential pathogenetic factor for human hypertension and kidney disease in humans.

Experimental models are extensively used to investigate the mechanisms responsible for the progression of renal diseases to end-stage renal failure. Besides conventional experimental models based on the toxic effect of drugs or on surgical removal of renal mass, rat strains that spontaneously develop kidney dysfunction have been used to identify the mechanisms that induce glomerular and tubular functional and structural changes (1). We and others have previously described renal functional and structural changes that develop in the MWF rat spontaneously with age (2–4); these animals have been selected from the Munich-Wistar strain by Frömeter for having a high number of surface glomeruli (5,6). They develop proteinuria at the early age of 10 wk, and by week 35 the kidney exhibits significant glomerulosclerosis (3,7). At 10 wk of age, systolic BP ranges from 140 to 150 mmHg and reaches approximately 180 mmHg when the animals are 9 mo old (8). Apart from this rather moderate hypertension, there are no other features of systemic disease that might be responsible for development of renal dysfunction, which manifest earlier in life than hypertension. As expected, in this strain, as in most other strains, female rats are protected from aging processes involving the kidney, with female MWF rats having lower urinary protein excretion than males of the same age (2,3).

It has been suggested that a renal abnormality that contributes to systemic hypertension is a reduced number of nephrons (9). A consequence of a low number of nephrons is a reduced ability to excrete sodium. Compensatory glomerular hemodynamic changes may develop to compensate this renal abnormality and may predispose to development of progressive renal function deterioration (10–12). The aim of this study was to investigate whether in the MWF strain the number of nephrons is reduced compared with the reference Wistar strain used as control.

To determine the number of nephrons, we used a maceration
The total number of glomeruli per kidney was then calculated using a design-based method, the disector/fractionator technique, to verify the reliability of glomerular number estimations obtained by kidney tissue maceration. In addition to the evaluation of glomerular number, we measured systolic BP and urinary protein excretion rate and estimated whole-kidney renal hemodynamic parameters and glomerular capillary tuft volume. These measurements allowed us to calculate key functional and structural parameters at the single-nephron level in male and female rats of both strains.

**Materials and Methods**

Male and female rats from two strains were used in this study: the MWF and the Wistar strains. Inbred MWF rats were bred and raised in our facilities (3). This colony of rats derives from animals originally provided by Dr. Hackbart from Hannover, Germany. In the past several years, our colony maintained constant features of previously described hypertensive, proteinuria, and glomerulosclerosis that developed spontaneously with age (2,14). Wistar rats were purchased from Charles River (Calco, Brescia, Italy). Animal care and experimental procedures in animals conformed to institutional guidelines that are in compliance with national and international laws and policies (European Economic Community Council Directive 86/609, OJ L 358-1, National Institutes of Health Guide for the Care and Use of Laboratory Animals).

Four groups of 20 animals, respectively male and female MWF rats and male and female Wistar rats, at 12 to 14 wk of age were used in this study. Before evaluation of glomerular number and/or kidney hemodynamics, protein excretion rate was determined using 24-h urine collections in metabolic changes, and awake systolic BP was determined using the tail-cuff method (15). Proteinuria was determined by the Coomassie blue G dye binding assay with bovine serum albumin as standard (16). The number of glomeruli per kidney was then measured using the maceration technique described previously (13) with the modifications of Bankir and Hollenberg (17). Briefly, animals were anesthetized by pentilea injection of thiopental sodium (10 mg/100 g body wt, pentothal sodium, Abbott Laboratories, Campoverde, Italy), placed on a constant temperature table, and tracheostomized. The left femoral artery was catheterized for blood sampling and for continuous monitoring of arterial pressure (AP) (Battaglia Rangoni, Bologna, Italy). A polyethylene catheter was inserted into the left femoral vein for infusion of 2 ml of a solution containing 5% Alcian blue 8GS (Sigma-Aldrich, Milano, Italy) dissolved in isotonic saline. The kidneys were then excised, decapsulated, weighed, and immersed in 1% ammonia for 30 min each. The right kidney was removed for maceration. The other kidney was then immersed in 1% ammonia for 30 min each. Blood samples were obtained from the femoral artery at the midpoint of each clearance period. Inulin and PAH concentrations in plasma and urine samples were determined as described previously (18,19). At the end of the clearance periods, animals were injected with Alcian blue, as described above, and the right kidney was removed for maceration. The other kidney was then fixed by perfusion, at the measured arterial pressure, with 1.25% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4. After fixation, two midcoronal sections of the kidney were post-fixed in Dubosq-Brazil fluid and embedded in paraffin. Sections 3 μm thick (Ultrrotome V, LKB, Sweden) were stained with Masson's trichrome, hematoxylin and eosin, and by the periodic acid-Schiff techniques. Glomerular volume (Vg) was determined as described previously (3,4,20), using a computer-based image analysis system (Power Macintosh 9600/350, Apple Computer, Cupertino, CA). Histologic sections were digitized from the microscope using a video camera (Panasonic, Matsushita Electric Co., Osaka, Japan) and displayed at final magnification of X1018, calculated from direct measurement of a reference grid. The outline of the minimal polygon around glomerular tuft area was manually traced, and its surface was automatically measured. Mean glomerular random cross-sectional area (Am) was determined in all glomeruli contained in a midcoronal section of each kidney. Mean value of Vg of individual animals was calculated using the formula Vg = (β/k) (Am)½, where k = 1.1 is a size distribution coefficient and β = 1.38 is the shape coefficient for the glomeruli (3).

To validate the estimates of nephron number obtained with maceration technique, we determined the glomerular number in three additional male MWF rats and in three male Wistar rats of 12 wk of age, using the disector/fractionator technique described previously (21-23), with slight modifications. The left kidney was perfusion-fixed with the glutaraldehyde solution, as described previously, removed, and cut in parallel slices of 1.5 mm thickness using tissue slicers. Every third slice was sampled (starting in random order with the first sampled slice), post-fixed in Dubosq-Brazil fluid, and embedded in paraffin. Each paraffin-embedded slice was entirely sectioned with a section thickness of 5 μm. Starting with a random order, series of 40 sections were separately collected, and the first and the fifth section of each series were mounted on glass slides and stained with periodic acid-Schiff. These two sections, later referred to as the sampling section and look-up section, respectively, were coded to allow blind estimation of glomerular number by the examiner (M. Bonassi). Tissue sections were examined on a Zeiss light microscope connected to the computer-based image analysis system. For systematic selection of the field of vision, the microscope table was equipped with two high-precision dial indicators mounted along two orthogonal directions, used by the examiner to move the microscope stage with predefined steps (1.0 ± 0.005 mm) in x and y direction. For each sampling section, a sequence of images was digitized with a ×10 objective and predefined steps in x and y direction with a field of vision of 0.711 mm². Color images were stored in the computer memory and subsequently analyzed using the computer program National Institutes of Health Image (version 1.60). Subsequently, the
look-up section was examined, using the reference of the two dial indicators for precise movement, to compare corresponding field of each individual image previously digitized from the sampling section. The two corresponding images were then compared, one on the camera monitor and the other on the computer monitor. Enlargement of the images was the same, because both images derive from the same video signal. Glomeruli that were present in the look-up section but disappeared in the sampling section and vice versa were counted. A fraction of cortical tissue used to perform glomerular counting was also determined to exclude areas of the renal cortex intersected by artificial edges. Using area/perimeter functions of the computer program, the area of all cortical tissue was measured in screen pixels \((A_s, A_c)\), together with the area of the cortex not intersected by artificial edges \((A_t)\). Finally, the glomerular number per kidney was calculated using the formula:

\[
N_g = 3 \times 10 \times 1.406 \times (A_s/A_t) \times (\Sigma Q^2/2)
\]

where 3 is the inverse of the slice-sampling fraction, 10 is the inverse of the section-sampling fraction, 1.406 is the inverse of the fieldsampling fraction, and \((A_s/A_t)\) is the fraction of kidney cortex used for glomerular counting.

**Statistical Analyses**

All results are expressed as mean ± SD. Statistical analyses were performed using the software package StatView (Abacus Concepts, Berkeley, CA). Data were analyzed using two-way ANOVA and linear regression analysis as appropriate. Significance level of differences between individual group means was established using the Bonferroni/Dunn procedure for multiple comparisons. Statistical significance level was defined as \(P < 0.05\).

**Results**

Mean body weight was significantly lower in MWF than in Wistar rats of either gender (Figure 1). In addition, in both strains body weight was considerably higher in males than in females. As shown in Figure 1, data of kidney weight paralleled those of body weight. Thus, the kidney weight/body weight ratio was numerically lower in male MWF rats than in females of the same strain (averaging 4.2 ± 0.6 and 3.8 ± 0.5 \(10^{-3}\), respectively), but the difference did not reach statistical significance. In Wistar rats, the kidney weight/body weight ratio averaged 4.5 ± 0.6 and 4.5 ± 0.8 \(10^{-3}\), respectively, in males and females, and these ratios were comparable to the corresponding ratios measured in the MWF strain. Systolic BP in awake animals was significantly higher in MWF animals (145 ± 8 and 145 ± 8 mmHg, respectively, in males and females) than in Wistar animals (averaging 131 ± 14 and 133 ± 8 mmHg, respectively, in males and females) (Figure 2). As expected from previous studies, proteinuria was already evident in male MWF rats (82 ± 24 mg/24 h) but not in female rats (13 ± 4 mg/24 h). Male and female Wistar rats also showed near-normal urinary protein excretions, averaging 18 ± 5 and 7 ± 2 mg/24 h, and the mean difference in protein excretion in males and females of the control strain was again significant.

Data on the number of nephrons and on glomerular volume are reported in Figure 3. We have preliminarily verified that no significant differences could be detected among determinations of the number of glomeruli in the same kidneys by different operators. We have also verified that estimations of glomerular number in two kidneys of the same animals did not differ significantly (data not shown). The number of glomeruli was found to be markedly and significantly lower in MWF animals than in the Wistar strain, for both male and female groups. Actually, in male and female MWF rats the mean number of glomeruli \((N_g/kidney)\) averaged, respectively, 13,690 ± 1,489 and 12,855 ± 1,781 gl/kidney, whereas in animals of the Wistar strain \(N_g/kidney\) averaged 26,955 ± 2,171 and 27,166 ± 1,754 gl/kidney. To take into account the large difference in body size of the four groups, we calculated the ratio of glomerular number to body weight in each animal (Figure 3). Male MWF rats had, on average, 88.4 ± 9.6 gl/g body wt, a value significantly lower than that observed in Wistar females (139.1 ± 19.7 gl/g body wt). In Wistar males, this ratio averaged 142.1 ± 14.0 gl/g body wt, or nearly 60% above the value in male MWF rats. In female Wistar rats, the number of glomeruli relative to body weight was significantly higher than in the other three groups, averaging 221.3 ± 22.1 gl/g body wt. An inverse correlation with high correlation coefficient \((r = 0.844)\) was found between the relative number of glomeruli \((N_g/g\ body\ wt)\) and the urinary protein excretion.
Figure 2. Systolic BP and urinary protein excretion measured in male and female MWF and Wistar rats at the age of 12 to 14 wk, before glomerular counting (n = 20 in each group).

Figure 3. Absolute number of glomeruli per one kidney and number of glomeruli per gram of body weight in male and female MWF and Wistar rats at the age of 12 to 14 wk (n = 20 in each group). In the bottom panel, glomerular tuft volume was measured in a subset of each group (n = 7).

The results of kidney hemodynamics evaluations are reported in Table 1. Mean absolute GFR was higher in male than in female rats of the same strain. Taking into account body weight, the relative GFR was also slightly but significantly higher in males than in females for both strains. Mean GFR/100 g body wt was comparable between males and females of the two strains. The same pattern was observed for absolute and relative RPF values. Filtration fraction was consistently lower in females than in males of both strains, but the differences did not reach statistical significance. By dividing the absolute values of GFR and RPF by the number of glomeruli in each animal of these groups, we calculated single-nephron GFR (SNGFR) and glomerular plasma flow (Qa). Mean SNGFR and Qa were significantly higher in MWF males than
Figure 4. Linear regression analysis of urinary protein excretion as a function of the number of glomeruli per unit of body weight ($N_g/g$ body wt, left panel) and of glomerular volume ($V_g$) as a function of $N_g/g$ body wt (right panel). For both parameters, a statistically significant correlation was found ($P < 0.01$; correlation coefficient $r = 0.844$ and 0.708, respectively, for urinary protein excretion and glomerular volume). Low number of glomeruli per unit of body weight was associated with higher urinary protein excretion and larger glomerular tuft volume; high number of glomeruli was associated with normal protein excretion and small glomerular volume.

in MWF females and Wistar rats of either gender (Table 1). Actually, SNGFR and $Q_a$ in Wistar males and females were approximately half of those calculated for MWF males and females, respectively.

Mean $V_g$ of animals that underwent renal hemodynamic evaluations is reported in Figure 3. Glomerular tuft volume was significantly higher in male MWF rats than in females of the same strain and in Wistar males. No significant differences were observed among $V_g$ in male and female Wistar rats, although female rats exhibited lower numerical values. Linear regression analysis showed a statistically significant inverse correlation ($r = 0.708$, $P < 0.01$) between the relative number of glomeruli ($N_g/g$ of body wt) and $V_g$ in the four groups (see Figure 4).

Discussion

The main finding of our investigation is that male MWF rats, which spontaneously develop proteinuria and glomerulosclerosis with age, have a lower than normal number of nephrons, much lower than observed in male Wistar rats. We made direct comparison between the results obtained with the maceration technique and the more modern, design-based disector/fractionator method, to exclude that the observed difference in the number of glomeruli between the two strains derives from technical problems eventually related to the maceration technique, such as loss and fragmentation of glomeruli, and difficulties in recognition of the capillary tufts. The results of the comparison show a rather good agreement between glomerular counts obtained with the two methods.

Besides the low number of nephrons, male MWF rats had a lower body and kidney weight than Wistar animals of the same age. This could possibly be attributed to a growth defect, likely related to the defect in growth hormone secretion reported previously (25) for the Munich-Wistar strain from which this strain originated. This growth abnormality may also lead to impaired nephrogenesis and thus may be responsible for the low number of glomeruli. Considering the relative number of nephrons per unit of body weight, male MWF animals have markedly fewer nephrons than females of this strain and also than Wistar controls. Actually, on average there are only 88 glomeruli per gram of body weight in the male MWF rat, whereas in the male Wistar rat there are more than 140 gl/g body wt. It is tempting to speculate that the reduced number of nephrons in this strain predisposes to the development of hypertension, proteinuria, and progressive glomerulosclerosis. We also observed a lower-than-normal absolute number of nephrons, and a growth defect in female MWF rats, which also develop proteinuria and glomerulosclerosis, albeit lower than in males of this strain (2,3). This is not in contrast with the above hypothesis, because, for the small body weight, MWF females had a relative number of glomeruli (139 gl/g body wt) that was 50% higher than that of males (Figure 4).

Because of technical difficulties, there is no extensive documentation on the number of nephrons in living humans, and this technique still relies on several assumptions (26). Glomerular number obtained with the disector/fractionator technique in kidneys from human autopsies shows that differences in gender and kidney mass tend to be higher in males than in females (27), and males have some 10 to 20% more nephrons than females (28). However, available data indicate that the number of nephrons in humans is characterized by a wide variability (27,29–31). It has also been suggested that those
Concomitant measurements of renal hemodynamic parameters and glomerular number allowed us to calculate SNGFR and afferent arteriolar plasma flow. These data show that glomerular capillaries of MWF males are hyperperfused and hyperfiltered. Actually, SNGFR and $Q_v$ values are almost twice the corresponding values calculated for male Wistar rats. This high rate of perfusion and filtration may derive theoretically from an increase in glomerular capillary pressure and ultrafiltration coefficient ($K_f$). We have previously reported that glomerular capillary pressure is normal in the MWF strain (2,8,24), and we favor the hypothesis that the glomerular hypertension in these animals must derive from an increased $K_f$. That $K_f$ can be elevated in MWF males is suggested by the observed enlargement of glomerular tuft in these animals, giving rise to an increase in filtering surface area.

Experimental and human data (reviewed in reference 9) suggest that reduced nephron number enhances systemic arterial pressure partly by an increase in renal sodium retention, which occurs when the total filtering surface area is low, unless sodium intake is considerably diminished. Even so, in the case of extreme reduction of renal mass, glomerular hypertension develops with increased plasma volume and low renin (10,32,33). Progressive decline of renal function in this setting is prompted by the concomitant effects of high systemic BP and altered renal hemodynamics, possibly finalized to restore to normal sodium excretion. This sequence of events might follow more or less sudden loss of nephron population, such as surgical ablation of renal mass; however, a different mechanism may underlie functional renal adaptation in the male MWF rat. In young animals, a slower process of adaptation may develop that does not require hemodynamic changes, but rather the involvement of structural adaptation of the glomerular capillary to achieve higher glomerular blood flow and filtration rates. This hypothesis would explain why glomerular capillary pressure is not abnormally elevated in these animals. Structural adaptations of the glomerular capillary tuft components suggest a major reorganization of the capillary network structure. We can speculate that structural adaptations would
elongate the capillary length to provide more filtering surface area. This can be obtained by elongation of capillary segments or by formation of new capillary loops, which, in any case, suggest reorganization of the glomerular structures. According to the observations of Kriz and coworkers (34–36), when the glomerular tuft is subjected to expansion, epithelial cells are not able to adapt adequately, because they do not divide, and existing cells must be stretched to cover a wider area of the glomerular capillary wall. This may result in focal damage of the interdigitating processes of the epithelial layer, structures that are responsible for water filtration and for the selective function of the membrane to circulating proteins (37,38). The resulting trafficking of plasma proteins across the glomerular wall, and along the tubules, may lead to further damage of the nephrons (39). Actually, uptake of large amounts of filtered proteins by tubules by endocytosis upregulates genes encoding inflammatory and vasoactive molecules, which serve to initiate an inflammatory reaction into the interstitium that is then followed by an accumulation of extracellular matrix and interstitial fibrosis (40,41).

In conclusion, the results of our investigation show that development of hypertension and spontaneous glomerular injury in male MWF rats is associated with an important reduction in the number of nephrons. Development of systemic hypertension and renal injury correlates with a low number of nephrons per unit of body weight, an index of the metabolic load of the nephrons. These results argue for the role of nephron number on the initiation and progression of kidney diseases in humans.

Acknowledgments
We thank Dr. L. Bankir for helpful discussion. We also thank Dr. G. Giovannetti and D. Cavallotti for excellent technical assistance. M. Bonassi helped in performing glomerular counting with the disector/fractionator method.

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