Nephrotoxin exposure in utero reduces glomerular number in sclerosis-prone but not sclerosis-resistant mice

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Nephrotoxin exposure in utero reduces glomerular number in sclerosis-prone but not sclerosis-resistant mice.

Background. We have previously found that nephron number was not fixed, that is, there was a direct correlation between low birth weight and decreased nephron number in infants. In sclerosis-prone rats, we found that gentamicin exposure in utero induced a reduction in glomerular number and aggravated glomerulosclerosis in adults. In mice, we found that an inborn 50% reduction in nephron number, caused by the Os mutation, was associated with glomerulosclerosis in sclerosis-prone (ROP1/1) mice, but not in sclerosis-resistant (C57BL/6J) mice. Because the genetic background determined the response to decreased nephron number, we asked whether the susceptibility changes in glomerular number and glomerulosclerosis were linked.

Methods. Gentamicin was administered before and after the onset of fetal nephrogenesis. (1) Prior to the onset of nephrogenesis, two groups of pregnant mice were treated from embryonic day (E) E8 to E12. In group A, early glomerular development was studied by placing ureteric ridges removed on E12 in vitro for four days, following which the ureteric bud branches and glomeruli were counted using lectin staining. In group B, nephron number was determined in spontaneously delivered 14-day-old (14PN) pups by counting glomeruli. (2) After the onset of nephrogenesis, to determine the direct effects of gentamicin on nephron induction, ureteric ridges were placed in organ culture at E12 of normal gestation, in the presence or absence of gentamicin. The number of glomeruli and ureteric bud branches were counted after six days in culture.

Results. A decrease in glomerular number and ureteric bud branches was observed in sclerosis-prone (ROP1/+) mice, irrespective of whether gentamicin was administered prior to or after the onset of nephrogenesis. Glomerular number and ureteric bud branching were not decreased by gentamicin in sclerosis-resistant (C57BL/6) mice.

Conclusions. These data provide evidence that there is a positive correlation between the susceptibility to glomerulosclerosis in adulthood and a reduction in nephron number in utero. Thus, exposure to nephrotoxins in utero compounds the risk of renal failure as an adult in sclerosis-prone individuals.

We have previously found that in studies of kidneys from victims of sudden infant death syndrome, nephron number and birth weight were inversely correlated [1]. These data suggested that nephron number is not fixed and could be modulated by in utero events. Because the number of nephrons in humans does not increase after birth, intrauterine renal events leading to a reduction in glomerular number is an important clinical issue. Oligomeganephronia, a disease with severe nephron deficit at birth, often leads to end-stage renal disease (ESRD) in infancy [2]. However, a recent study of 29 children with oligomeganephronia followed between 3 and 17 years showed that not all developed ESRD [3]. One reason for this discrepancy could be that the development of progressive glomerulosclerosis requires a permissive genetic background in addition to a reduction in nephron number.

We previously showed that glomerulosclerosis developed only in sclerosis-prone mice, data in agreement with that in children [4]. For these studies, we used mice with an inborn 50% reduction in nephron number caused by a radiation-induced oligosyndactylism mutation (Os), which has been back-crossed onto sclerosis-prone (ROP) and sclerosis-resistant (C57) mice. When ROP Os/+ and C57BL/6 Os/+ mice were subjected to unilateral nephrectomy in adulthood or were rendered diabetic, only ROP Os/+ mice developed glomerulosclerosis [5, 6]. Thus, the sclerotic response depended on the genetic background and was not stimulus specific.

We previously found that the glomerular number was decreased in pups of female sclerosis-prone (Sprague-Dawley) rats that had received gentamicin from embry-
Table 1. Data from mothers and pups (Protocol 1B)

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>BW E8</th>
<th>BW gain E8–12</th>
<th>Gestation time (h + 18 days)</th>
<th>Pups/litter</th>
<th>BIW/litter</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>5</td>
<td>23.8 ± 3.3</td>
<td>4.4 ± 0.6</td>
<td>31.2 ± 9.9</td>
<td>7.8 ± 1.6</td>
<td>1.37 ± 0.15</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>7</td>
<td>23.7 ± 1.9</td>
<td>3.2 ± 0.7 a</td>
<td>31.4 ± 9.9</td>
<td>7.0 ± 1.8</td>
<td>1.37 ± 0.06</td>
</tr>
<tr>
<td>ROP+/+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>5</td>
<td>24.9 ± 2.2</td>
<td>3.3 ± 1.1</td>
<td>24.1 ± 5.3</td>
<td>6.5 ± 1.9</td>
<td>1.18 ± 0.09</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>6</td>
<td>24.5 ± 2.3</td>
<td>0.5 ± 1.0 b</td>
<td>30.3 ± 6.6</td>
<td>6.3 ± 2.0</td>
<td>1.15 ± 0.07</td>
</tr>
</tbody>
</table>

Data are mean ± so. Abbreviations are: BW, body weight; BIW, birth weight.

Table 2. Data from pups used for glomerular counting at post-natal day 14 (PN14; Protocol 1B)

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>BIW g</th>
<th>BW g</th>
<th>KW mg</th>
<th>GN N × 10³</th>
<th>GN/KW N/mg</th>
<th>GN/BW N/g</th>
<th>KW/BW %</th>
</tr>
</thead>
<tbody>
<tr>
<td>C57BL/6</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>20</td>
<td>1.37 ± 0.15</td>
<td>7.7 ± 0.4</td>
<td>62.3 ± 7.5</td>
<td>13.9 ± 1.7</td>
<td>235 ± 22</td>
<td>1799 ± 224</td>
<td>0.84 ± 0.05</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>28</td>
<td>1.37 ± 0.11</td>
<td>7.5 ± 1.1</td>
<td>61.5 ± 4.2</td>
<td>14.1 ± 1.5</td>
<td>232 ± 27</td>
<td>1942 ± 563</td>
<td>0.79 ± 0.07</td>
</tr>
<tr>
<td>ROP+/+</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Saline</td>
<td>16</td>
<td>1.20 ± 0.09</td>
<td>7.2 ± 0.7</td>
<td>48.1 ± 4.1</td>
<td>12.3 ± 1.0</td>
<td>256 ± 22</td>
<td>1701 ± 132</td>
<td>0.67 ± 0.07</td>
</tr>
<tr>
<td>Gentamicin</td>
<td>10</td>
<td>1.21 ± 0.08</td>
<td>7.8 ± 0.6 a</td>
<td>52.9 ± 5.6</td>
<td>11.1 ± 1.6 a</td>
<td>211 ± 49 b</td>
<td>1430 ± 266 b</td>
<td>0.69 ± 0.08</td>
</tr>
</tbody>
</table>

Data are mean ± so. Abbreviations are: BIW, BW, KW, birth, body and kidney weight; GN, glomerular number. BW, KW and GN were assessed at PN14.

METHODS

Experimental protocols

Maternal treatment prior to the onset of nephrogenesis. (a) Effects on glomerular number and ureteric bud branching. Female 10- to 12-week-old C57BL/6 mice (Charles River, Paris, France) and ROP+/+ mice (Jackson Laboratories, Bar Harbor, ME, USA) were housed with males of the same strain overnight. The next day was defined as E0.

Pregnant females (17 C57BL/6 and 15 ROP+/+ mice) received 100 mg/kg gentamicin intramuscularly (gentamicin sulfate in saline) or an equal volume of saline from E8 to E12. This period overlaps the early stages of nephrogenesis in mice. At this time, there are no morphologically recognizable glomeruli. Injections were given into the thigh to avoid abdominal manipulation.

On E12, one group of gentamicin- (3 C57BL/6 and 2 ROP+/+ mice) and saline-treated females (two mice per strain) was anesthetized with sodium pentobarbital (60 mg/kg, intraperitoneally). The embryos were collected and weighed. Kidneys were removed aseptically, freed of exogenous tissue, and used in an organ culture system described previously for rats [8, 9]. Briefly, kidneys were placed in individual dishes on a 0.8 μm Millipore AA filter (Millipore, Saint-Quentin-en-Yvelines, France) floating on serum-free Dulbecco’s modified Eagle’s medium/Ham’s F12 (vol/vol) supplemented with 15 mM HEPES, 45 mM NaHCO₃ (pH 7.45 ± 0.05), 6.8 × 10⁻⁸ m selenium, 8.3 × 10⁻⁷ m insulin, 2 × 10⁻⁸ m triiodothyronine, and 7 × 10⁻⁹ m prostaglandin E₁. Transferrin-free medium was used to prevent further mesenchymoepithelial conversion, allowing us to restrict the analysis to those glomeruli induced at the time of explantation (E12) [10]. Kidneys were cultured for four days in a humidified incubator with 5% CO₂ at 37°C. The medium contained no antibiotics or fungicides and was changed daily. Glomerular number and ureteric bud branches were assessed by lectin staining.

(b) Effects on the final number of glomeruli. The second group of gentamicin- and saline-treated females (12 C57BL/6 and 11 ROP+/+; Table 1) was allowed to deliver spontaneously. Maternal weight and the duration of gestation were recorded. Pups were weighed within onic day (E) E8 to E12 [7]. A direct effect of gentamicin on nephron number was demonstrated using metanephros organ cultures [8]. In this study, gentamicin was chosen as an example of a nephrotoxin that can reduce glomerular number to determine whether the susceptibility to glomerulosclerosis and a reduction in nephron number were linked.
EXPERIMENTAL PROTOCOLS

1. Treatment in utero

![Diagram showing experimental protocols](image)

- Onset of nephrogenesis
- G
- Metanephros organ culture (effects on glomerular number and ureteric bud branching)
- Kidney maceration (effects on the final number of glomeruli)
- Birth

2. Treatment in vitro

![Diagram showing experimental protocols](image)

- Onset of nephrogenesis
- G
- Metanephros organ culture (effects on glomerular number and renal growth)

10 hours following birth and were individually marked. The litter size was randomly reduced to eight pups. On PN14, pups were weighed and killed with an overdose of sodium pentobarbital. The left kidney was removed and weighed, and preparation for glomerular counts commenced the same day (Table 2).

Treatment after the onset of nephrogenesis. Effects on glomerular number in culture. Untreated pregnant females (three C57BL/6 and four ROP+/+) were anesthe-tized at E12. Kidneys were removed and grown for six days in a medium described earlier in this article with the addition of \(6.2 \times 10^{-8}\) m transferrin. This medium supports normal organogenesis and metanephros differentiation [8–10]. One kidney was cultured in the presence of 10 \(\mu g/ml\) gentamicin. The contralateral kidney was cultured in normal medium and served as an internal control. The use of paired experiments has been found to reduce interassay variability because the in vitro growth of embryonic kidneys varies between embryos of the same litter. The glomerular number was assessed following lectin staining. All reagents were purchased from Sigma Chemical Company (St. Louis, MO, USA).

A summary of the experimental protocols is shown in Figure 1.
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Fig. 2. Lectin histochemistry. Following gentamicin (B and D) or saline (A and C) treatment of the mothers from gestation E8 to E12, embryonic kidneys were placed in culture for four days (Protocol 1A). Glomerular and tubular segments were visualized by PNA and HPA coupled to the same fluorochrome. Gentamicin treatment did not affect glomerular number or ureteric bud branching in C57BL/6 kidneys (B vs. A). By contrast, a decrease in glomerular number and ureteric bud ends was observed in gentamicin-treated ROP+/+ kidneys (D vs. C). No glomeruli were found in lateral areas (arrows; bar represents 300 μm).
branches was performed by two observers. All of the glomeruli or ureteric buds were visualized in each specimen by focusing through the specimen [8]. This was accomplished by moving the stage so that all fields from the bottom of the specimen to its most superficial parts were examined. The results are means of the four counts.

Determination of glomerular number in postnatal (PN14) kidneys

The kidney was decapsulated and macerated in 50% HCl for 30 minutes at 37°C. After rinsing with tap water, the brei was stored overnight at 4°C. The flask was gently shaken on the following day. The tissue was homogenized with a glass stirring rod, and the tubules and glomeruli were suspended in 25 ml of tap water. Glomeruli were counted in two different 500 µl aliquots by each of two investigators. The total number of glomeruli was calculated using the mean of the four counts.

Statistical analysis

The Mann-Whitney test was used to evaluate differences between kidneys of embryos from treated and untreated mothers (Protocol 1A). Treated and untreated females of each strain and their offspring (Protocol 1B) were compared by the unpaired Student’s t-test. Pairs of kidneys in each strain treated in vitro (Protocol 2A) were compared by Wilcoxon signed rank test. All data were expressed as mean ± sd. The significance level was set at P < 0.05.

RESULTS

Maternal gentamicin treatment prior to the onset of nephrogenesis

Effects on glomerular number and ureteric bud branching. Transferrin-free medium prevents the further induction of glomeruli in vitro. Thus, any glomeruli identified had been induced at the time of explantation.

After in utero exposure to gentamicin or saline, the kidneys did not have further drug exposure. Gentamicin-treated ROP+/+ kidneys had fewer glomeruli than their saline-treated controls (36% decrease, P < 0.05; Figs. 2A and 3A). The decrease in glomerular number was restricted to the lateral areas of gentamicin-treated ROP+/+ kidneys (Fig. 2D). In contrast, the number of glomeruli found in gentamicin-treated C57BL/6 kidneys was identical to that in saline-treated controls.

There was a 20% decrease in the number of ureteric buds in gentamicin-treated ROP+/+ kidneys (P < 0.05; Figs. 2 and 3B). In contrast, gentamicin did not affect the number of ureteric bud branches in C57BL/6 kidneys. Gentamicin treatment did not affect the elongation of existing ureteric buds in ROP+/+ or C57BL/6 kidneys.

Effects on the final number of glomeruli. Body weight was similar in both strains at treatment onset (Table 1).
Gentamicin-injected $ROP^{+/+}$ mice gained much less weight from E8 to E12 than did saline-injected $ROP^{+/+}$ mice ($0.5 \pm 1.0$ vs. $3.3 \pm 1.1$ g, $P < 0.001$). In contrast, gentamicin-injected C57BL/6 females gained only slightly less weight than did saline-injected C57BL/6 females during this same period of gestation ($3.2 \pm 0.7$ vs. $4.4 \pm 0.6$ g, $P < 0.01$).

Gentamicin treatment did not influence gestation time, number of pups/litter, or birth weight/litter in either strain. The frequency distribution of birthweights did not differ between treatment groups or mouse strains (data not shown). Among saline-treated litters, one $ROP^{+/+}$ litter died (mean birthweight $1.05$ g). No increase in perinatal mortality was observed in gentamicin-treated C57BL/6 litters. However, three of six gentamicin-treated $ROP^{+/+}$ mothers cannibalized their litters. The mean birthweight was low in two of the cannibalized litters ($<1.10$ g). The death of the third gentamicin-treated litter appeared to be related to a prolonged (16 hr) delivery. The resultant loss of pups among $ROP^{+/+}$ litters of gentamicin-treated mothers resulted in a reduction in the number of pups available for glomerular counting.

Four mice per litter were randomly selected for glomerular counting at PN14 (Table 2). Gentamicin-treated $ROP^{+/+}$ pups had a higher body weight (BW) and kidney weight (KW) and lower glomerular numbers (GN; $11.1 \pm 1.6 \times 10^3$ vs. $12.3 \pm 1.0 \times 10^3$, $P < 0.05$, 10% decrease) at PN14 than saline-treated $ROP^{+/+}$ pups. The GN/KW ratios ($211 \pm 49$ vs. $256 \pm 22$ number/mg) and GN/BW ($1430 \pm 266$ vs. $1701 \pm 132$ number/g) were also significantly decreased in the gentamicin-treated $ROP^{+/+}$ group compared with the saline-treated $ROP^{+/+}$ group ($P < 0.01$). In contrast, there were no differences between saline-treated and gentamicin-treated C57BL/6 pups with respect to birthweight, BW, KW, GN, GN/KW, and GN/BW and KW/BW ratios at PN14.
examining nephron development in vitro. A post-treatment reduction of glomerular number was found in ROP+/+ mice irrespective of whether or not gentamicin was administered prior to or after the onset of nephrogenesis.

Nephrogenesis begins at approximately E12 and is not complete until a few days after birth in mice [12]. The initial stage of nephron formation was targeted in this study by starting gentamicin administration at E8. Because we previously found that low birthweight was associated with nephron reduction, gentamicin administration was limited to five days to avoid intrauterine growth retardation [13].

The reduction of glomerular number and ureteric bud branches observed in vitro in ROP+/+ kidneys was restricted to the polar areas (Fig. 2D). Nephrons are first induced and attached to buds in the midpolar area, whereas in polar areas, nephron induction occurs later [14]. Our findings suggest that gentamicin altered ureteric bud branching rather than nephron induction in ROP+/+ mice. This is consistent with our previous findings in rats [11]. The reduction of glomerular number induced by gentamicin in ROP+/+ mice was mediated by a direct effect on ureteric bud branching. This reduction occurred if gentamicin was started either before or after the onset of nephrogenesis. Thus, the risk of developing a reduction in glomerular number following intrauterine exposure to renal toxins is not limited to the initial phases of renal development and may continue until ureteric bud branching is complete.

Gentamicin-treated ROP+/+ females gained significantly less weight than did their saline-injected controls from E8 through E12. However, gentamicin-treated C57BL/6 females gained only slightly less weight than their saline-treated controls during this period. This suggests that, although maternal gentamicin toxicity is present in both strains, it is more pronounced in ROP+/+ mice. The gestation time was normal in both gentamicin- and saline-treated mice of both mouse strains in this study. These data differ from that in the Sprague Dawley rat, in which gestation time was shortened by gentamicin treatment [7].

The birthweight of gentamicin-treated and saline-treated ROP+/+ pups was identical. Therefore, the reduction in glomerular number in gentamicin-treated ROP+/+ mice was likely a direct gentamicin effect and could not be ascribed to a change in birthweight. The pups of gentamicin-treated ROP+/+ mice, which were subsequently cannibalized, had lower birth weights than saline-treated controls. Because low birthweight and decreased nephron number are linked, the nephron number in these mice might be expected to be reduced compared with saline-treated controls [13]. Therefore, the loss of data from these pups would be expected to bias...
the data toward an underestimation rather than an exaggeration of a gentamicin-induced reduction of glomeruli.

We found that gentamicin treatment reduced the glomerular number in ROP+/+ mice by 10 to 36%. This was considered to be a functionally significant change because a 20% decrement in glomerular number was sufficient to accelerate the development of glomerular lesions in adult Sprague-Dawley rats [15]. Because ROP+/+ mice are glomerulosclerosis prone, a comparable nephron reduction most likely constitutes an important additional renal risk factor in this mouse strain.

Nephrogenesis in vitamin A-deficient Sprague-Dawley rats is impaired, evidence that in utero metabolic events may also influence nephron number [16]. The impact of an abnormal in utero metabolic environment has recently been studied in Pima Indians [17]. There was a positive association demonstrated between the presence of type 2 diabetes in the mother during pregnancy and the development of an elevated urinary albumin excretion rate in adulthood.

In summary, these data show that a mouse strain susceptible to glomerulosclerosis develops a decrease in glomerular number following intrauterine exposure to renal toxins. Because a reduction in glomerular number is an additional stimulus to glomerulosclerosis, prenatal exposure to renal toxins may compound the hazard of developing renal failure in sclerosis-prone individuals.

References

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