Gentamicin Administered during Gestation Alters Glomerular Basement Membrane Development

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Gentamicin during gestation alters glomerular basement membrane development. A drug-induced nephrotoxicity was described for neonates after gentamicin was given intraperitoneally to pregnant Wistar rats; glomerular alterations and changes in permselectivity were important. We investigated the ultrastructure of the glomerular basement membrane (GBM), the arrangement of anionic sites, and the urinary proteins at two ages, with 1-day- and 12-month-old control and prenatally exposed animals. For neonates, the pattern of glomerular differentiation was similar, anionic sites were made of heparan sulfate proteoglycans, and the GBM had the same total thickness in both groups. After transplacental gentamicin exposure, the lamina densa was larger; the laminae rarae were thinner; the density of anionic sites was increased; the levels of hydroxyproline, sulfate, and hexuronic acid in the kidney were increased; and the immunoelectrophoresis of urinary proteins was abnormal. For adults, prenatal exposure to gentamicin led to altered juxta-medullary glomeruli with a larger GBM and abundant anionic sites, especially in the lamina densa, and to a protein excretion different from that of controls. Thus, gentamicin administered during pregnancy leads to permanent alterations of the GBM with modifications of both the layers and the anionic sites, possibly because of a perturbed protein metabolism. These altered glomeruli are at risk during life and could be the starting point for a kidney disease.

Analysis of drugs in relation to their safety for mother and fetus is usually restricted to gross examinations of the newborn alone. The developing kidney, considered to be resistant to nephrotoxicity (17), is rarely investigated despite the fact that renal alterations in young and puppies have been described after administration of different drugs (18, 37). Only recently has there begun to emerge a perception of how alterations of the developing kidney could explain a large variety of renal disorders, especially glomerular ones (3, 44, 50).

In contrast, for adults nephrotoxicity studies have been expanding for 2 decades mainly because kidney diseases constitute an important economic burden which could be relieved in part by prevention. Following the widespread use of aminoglycoside antibiotics, gentamicin-induced nephrotoxicity became one of the most investigated drug-induced renal injuries (7, 22, 24, 29, 43).

Observations of in utero aminoglycoside-induced nephrotoxicity after antibiotics had been given to pregnant rats (20, 21, 31–33) showed that, contrary to what is seen with adults, glomerular modifications are very common while tubules are less altered. Also, an abnormal proteinuria was described and a marker with a high molecular weight (anionic ferritin) was found in the urine and glomerular podocytes after being injected intravenously into neonates (42), indicating an altered permselectivity of the glomerular basement membrane (GBM) of the mature glomeruli.

Renal maturation, similar in all mammals, is often termined before birth (45), but not in the rat, which completes its nephrogenesis in the first 2 weeks of life, thus making possible its observation. The GBM is progressively formed by two membranes: the lamina rara interna (LRI; formed from the endothelial cells) and the lamina rara externa (LRE; formed from the pedicles of the epithelial cells), which fuse and generate a common lamina densa (LD) in the central part (2, 19, 40, 44). In the rat neonate, all these stages can be observed, the first differentiated (older) nephrons being in the juxta-medullary area. The urinary is formed in mature glomeruli by filtration through the GBM, which stops the long molecules, such as proteins (12). This permselectivity of the GBM is considered to be mainly due to an electrostatic shield made of highly negatively charged heparan sulfate proteoglycans constituting the anionic sites situated in the LRI and LRE (13, 25, 26, 39).

The present study was designed to investigate whether a modification of the layers and/or the anionic sites of the GBM could account for the altered permselectivity.

We verified that in prenatally gentamicin-exposed neonates the anionic sites of the GBM were made of heparan sulfate proteoglycans. Quantifying the GBM layers and anionic sites and dosing components of the extracellular matrix, we found that the developing GBM was abnormal in the treated neonates.

Finally, we have found that morphological and functional alterations of the GBM were still present in the juxta-medullary glomeruli of the adult animals, i.e., 12-month-old rats, prenatally exposed to gentamicin, thus making this observation of clinical relevance.

(A preliminary account was presented as an abstract at the
Materials and Methods

Animals and treatments. Female Wistar rats were mated during the night. The day that sperm was found in the vaginal smear was considered to be day 0 of gestation. Pregnant rats were then housed individually in plastic cages and fed a standard diet (UAR113); water was allowed ad libitum. Gentamicin (Gentalline; Laboratoire Unicet, Paris, France) was injected intraperitoneally (75 mg/kg of body weight) into the treated group, and a similar volume of saline was administered to the control group at 4:00 p.m. on days 7 to 11 (organogenesis) and 14 to 18 (beginning of nephrogenesis). For investigations with neonates, 8 mothers constituted the treated group (84 neonates) and 7 constituted the control group (79 neonates). Deliveries occurred normally on day 21 of gestation. The body weight and the blood biology of the mothers were routinely checked; no significant differences between groups were observed, as previously reported (31). Neonates were studied the following day.

Adult rats (12 months old) came from litters designed for a former study using the same protocol of treatment but in which neonates were differentially investigated (42). The rats from these litters had been prenatally exposed to gentamicin; five rats, each one from a different litter, were taken for this study, along with four control rats, also each from a different litter.

Disclosure of anionic sites by polyethyleneimine or cationized ferritin (38, 41). Polyethyleneimine (Sera; molecular weight, 30,000) was injected intravenously (5 mg/g of body weight in 10 μl of saline) into gentamicin-exposed rats (19 neonates and 5 adults) and controls (12 neonates and 4 adults). Thirty min later, kidneys were decapsulated and fixation was initiated by dripping 0.1% glutaraldehyde–2% phosphotungstic acid in sodium cacodylate buffer (0.12 M, pH 7.4) containing 0.043 M NaCl for 10 min. Kidneys were then excised, and prefixed cortex sections were immersed for 3 h in 1% glutaraldehyde–2% phosphotungstic acid in the same buffer at room temperature.

Cationized ferritin, pH 7.4 (Miles), was injected intravenously (0.3 mg/g of body weight in 10 μl of saline) into neonates (nine prenatally exposed rats and seven controls). After 30 min, kidneys were excised and sections were fixed in 2% glutaraldehyde–2% paraformaldehyde in sodium cacodylate buffer (0.12 M, pH 7.4) plus 0.043 M NaCl for 3 h at room temperature.

In both cases, kidney sections were postfixed in ferricyanide-reduced osmium for 90 min at room temperature, dehydrated in graded ethanol, and embedded in Epon. Ultrathin sections were stained with uranyl acetate after polyethyleneimine treatment and with bismuth nitrate after cationized ferritin treatment.

Morphometric studies of GBM and anionic sites. Blinded observations were made at random for kidneys from one animal of each litter (eight prenatally gentamicin-exposed neonates and seven controls). A minimum of three mature glomeruli per animal and five GBMs per glomerulus were studied. Only sections in which slit membranes and endothelial pores appeared transversally cut, i.e., cross-sections, were considered.

Data were obtained from a computer-based analysis of a manually drawn picture (Biocom System) superimposed on a video image of glomerular areas (magnification range, ×30,000 to ×45,000). The thickness of the total GBM and of each of its three layers (LD, LRE, and LRI) was measured.

Anionic sites disclosed by polyethyleneimine were counted; the results were expressed as density of anionic sites per square micrometer of their containing layer (LRI or LRE).

Statistical analysis was performed according to the Mann-Whitney test.

Histochemical study of anionic sites by enzymatic digestion (two neonates per litter). Methods of fixation were developed according to MacLean and Nakane (30). Briefly, after fixation in 2% paraformaldehyde–0.75 M lysine–0.01 M sodium periodate in 0.037 M phosphate buffer solution for 4 h at room temperature, tissues were washed in phosphate buffer and then in graded cryoprotective medium containing dimethyl sulfoxide and sucrose in phosphate buffer. Sections (30 μm) were cut with a cryostat microscope (American Optical Corporation), washed in 0.2 M sodium cacodylate buffer (pH 7.4), and incubated with enzymes.

Heparitinase and chondroitinase ABC (Miles) were used at the concentration of 1 U/ml of incubation medium. Frozen sections (30 μm) were incubated for heparitinase in 0.15 M phosphate buffer, pH 7.0, and for chondroitinase ABC in 0.05 M Tris buffer, pH 7.0. They remained in the enzymatic medium for 12 h at 37°C and then were washed in the appropriate buffer, incubated again overnight with constant agitation in 1% polyethyleneimine (Polyscience; molecular weight, 1,200–7% sucrose in the same buffer at 4°C, washed twice in sodium cacodylate sucrose buffer, stained and fixed with 2% phosphotungstic acid–1% glutaraldehyde, and then postfixed in ferrocyanide-reduced osmium and embedded in Epon.

Controls consisted of sections incubated in the same buffer without enzymatic treatment.

Doses of extracellular matrix components (two neonates per litter). Since the neonate kidney is only partly mature, it was impossible to dissociate the glomeruli by a conventional sieving technique; therefore, we used a homogenate of the whole kidney.

(i) Proteins. Kidneys were hydrolyzed in 0.5 N NaOH at 60°C for 3 h, and protein content was determined by Lowry's technique (28).

(ii) Collagens. After acidic hydrolysis of the kidney homogenate in 6 N HCl at 100°C for 48 h, collagen was assessed on the basis of hydroxyproline content measured according to Bergman and Lockley (9).

(iii) Sulfate and hexuronic acid. Kidneys were hydrolyzed in 4 N HCl at 100°C for 48 h, and then sulfate was measured according to Terho and Hartilia (47) and uronic acid was measured according to Bitter and Muir (10).

Each sample of hydrolysate was dried by rotary evaporation to eliminate all remains of HCl and then dissolved in distilled water.

Immunoelectrophoretic analysis of urinary proteins. Urine samples from neonates were fractionated by electrophoresis in agar gel (Difco Laboratories) at 1% in Veronal buffer, pH 8.2, and then subjected to immunoelectrophoresis with specific anti-rat serum. Sera from healthy rats were used as controls.

Urine from 12-month-old rats was first dialyzed for 24 h against distilled water and then concentrated 10 times before immunoelectrophoresis.

The electron microscope used throughout was a Philips 201 (Service d'accueil du Centre National de la Recherche Scientifique, Paris, France).
RESULTS

Neonates. The duration of the gestation and the mean number of neonates per litter were similar in both treated and control groups. As compared with those of the controls, the body weights (controls, 6.95 ± 0.005 g, n = 79; treated, 6.32 ± 0.07 g, n = 84, P < 0.01) and the kidney weights (39.4 ± 0.006 mg; 34.02 ± 0.8 mg, P < 0.01) were significantly decreased in prenatally gentamicin-exposed neonates; the kidney-to-body-weight ratio was not different (5.66 ± 0.006 versus 5.58 ± 0.33, not statistically significant).

Because of the postbirth renal maturation in the rat, all steps of the nephron differentiation, from the earliest stages in the subcapsular region to the fully mature glomeruli in the juxta-medullary area, were seen.

In treated neonates, the proximal tubular cells presented the lesions previously reported (31); they were not investigated further. The glomeruli presented no necrosis, but the epithelial cells were frequently altered: the lysosomes contained myeloid bodies, the mitochondria contained phospholipid deposits, the Golgi apparatus's cisternae were locally swollen, and next to these areas, membranes appeared closely packed, resembling small myeloid bodies.

Compared with those of controls, the developing GBM seemed abnormal in the treated animals; in the early stages of maturation, the fibrillar material appeared more important in the LRE and LRI before they joined to form the LD (Fig. 1a and c); in later stages, the LD itself looked larger and denser. The anionic sites, disclosed either by polyethyleneimine (Fig. 1) or cationized ferritin (Fig. 2), were apparently more numerous in the LRE and LRI.

Morphometric analysis, performed on the most differentiated glomeruli (juxta-medullary area), showed similar thicknesses of the GBM in both groups of neonates (controls, 55.5 ± 0.36 nm; treated, 52.2 ± 0.26 nm, not statistically significant). Nevertheless, the GBM layers were different from those of controls in treated neonates: the LRI and LRE were thinner (LRI: controls, 14.2 ± 0.09 nm, and treated, 10.3 ± 0.07 nm, P < 0.01; LRE: 14.3 ± 0.09 nm versus 10.3 ± 0.06 nm, P < 0.01) while the LD was enlarged (controls, 25.9 ± 0.21 nm; treated, 31.7 ± 0.19 nm, P < 0.001). The counting of anionic sites showed an increase of their number per square micrometer of the LRE or LRI and thus an increased density (LRI: controls, 277.54 ± 20.72 U/μm²; treated, 615.60 ± 48.0 U/μm², P < 0.001; LRE: 464.63 ± 26.34 U/μm² versus 832.98 ± 59.78 U/μm², P < 0.001).

Histoenzymatic studies revealed that, in both groups of neonates, the anionic sites were removed by heparitinase while they were unaffected after chondroitinase ABC (Fig. 3).

Extracellular matrix components, i.e., hydroxyproline level reflecting collagen content and sulfate and hexuronic
acid level reflecting glycosaminoglycans, were enhanced following gentamicin exposure as shown in Table 1.

Sulfate-to-hexuronic acid and hexuronic acid-to-hydroxyproline ratios were increased (+17 and +30%, respectively), indicating that glycosaminoglycans were more sulfated and quantitatively more abundant than in controls.

Immunoelectrophoresis with anti-rat serum showed for controls only one precipitation bow of albumin whereas for treated animals other proteins, recognized by antibodies raised against whole-rat-serum proteins, were also observed (see Fig. 5).

Adults. In 12-month-old rats prenatally exposed to gentamicin, the GBM was always larger than in controls. Anionic sites were abnormally arranged: while in controls the usual feature of numerous anionic sites in the LRE and LRI was observed, in prenatally exposed rats anionic sites were seen throughout the GBM whatever the layer (Fig. 4).

Immunoelectrophoresis with anti-rat serum showed, for 12-month-old rats prenatally exposed to gentamicin, a very large precipitation bow corresponding to albumin and maybe to other proteins; this bow was always more abundant than that for controls (Fig. 5).

**DISCUSSION**

By giving a drug to the mother before birth, we observed a drug-induced alteration of the GBM in the developing kidney; this modification is still present at adult age and is of clinical relevance since a pathological forming of basement membranes is suspected in several kidney diseases (3, 48, 50).

The dosage of the drug was calculated to allow passage into all the feto-placental units while being given for a few days only. In this model, the mothers were never clinically affected by the treatment and the blood chemistry was not significantly different from that of controls (31, 33). In addition, it should be noted that in rats the number of nephrons per unit of weight is around 12 times greater than in humans while the nephron size is only slightly smaller; thus, the surface for reabsorption should be notably larger. Moreover, the value of a given glomerular filtration rate in rats could be calculated differently, and the result would thus vary between one and four times, depending on the use of body weight or body surface reference; also, the extracellular fluid is filtered at least four times in rats while it is filtered only once in humans during the same period of time (46).
Therefore, the drug dosage may be different and should be related to species and clinical effect.

In the present study, the previously reported low body weight and morphological features of this model (31, 33, 42) were observed: as usual, the common tubular alterations due to the gentamicin toxicity were less important than in adults, and frequent glomerular alterations were seen with myeloid bodies (or myelin figures) and autophagic vacuoles in epithelial cells. Since with this glomerulotoxicity an altered permselectivity has been established (42), we focused on GBM alterations.

We found that after prenatal exposure to gentamicin, the GBM layers had a different thickness; in particular, the LD was larger in neonates and in adults, the anionic sites had an increased density in neonates and were more numerous and mainly dispersed in the LD in adults, and the extracellular matrix components were increased.

Morphological alterations of the GBM were noted for all stages of maturation: at developmental stages, before their fusion, the LRE and LRI were larger than those in controls (Fig. 1a and c); in the mature GBM, with the LRE and LRI forming a common LD, the layers were abnormal, the LD being larger and the LRE and LRI being thinner than those in controls (Fig. 1b and d). Quantification in neonates was done with mature glomeruli since laminae rarae were irregular and difficult to clearly delineate in the forming glomeruli. The observed values of GBM thickness in neonates were different from those known for adults because kidney growth was unfinished. In adult 12-month-old rats, these alterations were evident and seen in the juxta-medullary area only, i.e., the older nephrons (Fig. 4); glomeruli formed after the prenatal exposure were normal.

To explain the functional alterations seen with this model, i.e., proteinuria and crossing of the anionic ferritin through this abnormal GBM (42), a change in the anionic sites also could be supposed (8, 27). Proteinuria has been associated with a decrease in the concentration of the anionic sites (11, 14–16, 34, 35, 49). In the present study, the density of anionic sites per square micrometer of the LRE and LRI was increased; this could be explained in part by the low thickness of the LRE and LRI causing the anionic sites to be situated closer together. The influence on these results of a possible polyethyleneimine-induced change in the GBM as it was reported (4) is unlikely since studies were done comparatively for both control and treated neonates.

An altered protein metabolism giving a decrease in activity of key proteolytic enzymes following direct gentamicin exposure has been reported for adult kidneys (36). Gentamicin-induced glomerulotoxicity has been well described from functional measurements of adults (5, 6). In our study of transplacental gentamicin-induced glomerulotoxicity in neonates, assessed by morphology and functional studies, an altered protein metabolism was likely in glomerular cells.

**TABLE 1. Extracellular matrix components in kidneys of control and prenatally gentamicin-treated neonates**

<table>
<thead>
<tr>
<th>Component(s) or ratio</th>
<th>Amt of component or ratio for neonate group*</th>
<th>Control</th>
<th>Gentamicin-treateda</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sulfates</td>
<td>5.4 ± 0.01</td>
<td>7.9 ± 0.01*</td>
<td></td>
</tr>
<tr>
<td>Hydroxyproline</td>
<td>1.14 ± 0.01</td>
<td>1.61 ± 0.03*</td>
<td></td>
</tr>
<tr>
<td>Hexuronic acid</td>
<td>3.5 ± 0.07</td>
<td>4.5 ± 0.07*</td>
<td></td>
</tr>
<tr>
<td>Sulfate/hexuronic acid ratio</td>
<td>1.54</td>
<td>1.79</td>
<td></td>
</tr>
<tr>
<td>Hexuronic acid/hydroxyproline ratio</td>
<td>3.21</td>
<td>4.18*</td>
<td></td>
</tr>
</tbody>
</table>

* There were 15 neonates in the control group and 16 in the gentamicin-treated group. Values are expressed as micrograms per microgram of protein.

± mean ± standard error of the mean.

a Asterisk indicates that "P < 0.05.

FIG. 3. Histochemical characterization of anionic sites in differentiated GBM (magnification, ×70,000). See text for details. (a and d) Untreated sections (O). (b and e) Sections treated by heparitinase (panel b, HEP) or chondroitinase ABC (panel e, CH) from neonates prenatally exposed to gentamicin (T). (c and f) Sections treated by heparitinase (panel c, HEP) or chondroitinase ABC (panel f, CH) from control neonates (C). US, urinary space. Bar, 0.05 μm.
GENTAMICIN ALTERS GBM DEVELOPMENT

FIG. 4. Localization of anionic sites by polyethyleneimine in 12-month-old rats (magnification, ×60,000). (a) Control. (b) Prenatally exposed to gentamicin. CAP, capillary lumen; P, podocyte; US, urinary space. Arrows show polyethyleneimine deposits in laminae rarae; arrowheads show polyethyleneimine deposits in the LD. Bars, 0.5 μm.

Generating type IV collagen proteins of the GBM layers (50). While histoenzymatic studies demonstrated that anionic sites were similarly made of heparan sulfate proteoglycans in controls and in treated neonates (Fig. 3) (1), modifications of the contents of GBM components in kidneys from treated neonates were observed. The increase in hydroxyproline content, a component of collagen, and the increases in sulfate and hexuronic acid, involved in the glycosaminoglycan production, imply a modified metabolism of both the network and the anionic sites of the GBM. The former could account for the larger LD in neonates and adults and may explain in part the altered permselectivity while the latter could account for the larger density of anionic sites. Moreover, sulfate and hexuronic acid were more increased than hydroxyproline, indicating a constitution of anionic sites more exaggerated than the collagen production, therefore explaining the aspects of GBM in adults with anionic sites dispersed in the three layers and abundant in the LD.

Thus, it is possible to reconcile the observed morphological changes and the altered permselectivity: an abnormal network of the three layers of the GBM together with anionic sites modified in their formation and/or disposition could form a less efficacious barrier against protein filtration.

If such a large protein as anionic ferritin is able to cross the altered GBM, the blood proteins could be lost in the same way. Indeed, the anionic ferritin has been seen in urine and
in podocytes (42). Immunoelectrophoresis of urine from treated neonates showed the presence of proteins which were not observed in urine from controls (Fig. 5). Moreover, the albuminuria was larger in urine from prenatally exposed adults than in that from controls. Therefore, a subtle urinary loss of proteins together with an altered protein metabolism could account for the low body weight of prenatally gentamicin-exposed neonates, which was always lower than that of controls. However, reduced feeding of treated mothers cannot be excluded; it seems unlikely since, as usual in this model, the mothers were clinically and biologically unaffected.

Would an altered GBM in a relatively small area in adult kidneys be clinically relevant since the overall renal function could be assumed to be satisfactory from the large number of nephrons developed after the drug administration? The altered nephrons could be more sensitive to aging or to attack during life and therefore be the starting point of an acquired renal disease. In effect, such a possibility arises from observations that in children the juxta-medullary glomeruli are more likely to be affected by acquired focal and segmental glomerulosclerosis compared with more peripheral glomeruli (3, 23). Also, proteoglycan sites were abundant in adults' LD and they are often involved in pathological situations, especially in proteinuria and/or immune complex diseases (48); of interest is the abnormal protein immunoelectrophoresis we noted for the prenatally exposed adults.

It should be emphasized that the developing kidney does not escape drug-induced toxicity, and drugs could be harmful through placental transfer. Moreover, it is conceivable that other commonly used drugs could, like gentamicin, lead to alterations of the GBM that are potentially harmful but imperceptible by the usual examinations. Clearly, this area of investigation needs further studies with respect to drug prescription safety.

In conclusion, after gentamicin administration during pregnancy, the maturation of the GBM was altered, leading to permanent modifications during life: the layers of the GBM (LD, LRE, and LRI) were abnormal, suggesting a modified network possibly due to an altered protein metabolism; anionic sites, made of heparan sulfate proteoglycans as in controls, presented perturbed density and localization; finally, a defect in glomerular permeability (abnormal proteinuria) was seen for neonates and for adults.

Therefore, the maturation of basement membranes could be modified by a transplacental drug-induced toxicity. The juxta-medullary glomeruli involved in this prenatal exposure are also those which were demonstrated to be more at risk in children.

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