Evidence for Splicing New Basement Membrane into Old during Glomerular Development in Newborn Rat Kidneys

Dale R. Abrahamson and Elizabeth W. Perry
Department of Cell Biology and Anatomy, University of Alabama at Birmingham, Birmingham, Alabama 35294

Abstract. Tannic acid in glutaraldehyde fixatives greatly enhanced the visualization of two developmentally and morphologically distinct stages in glomerular basement membrane (GBM) formation in newborn rat kidneys. First, in early stage glomeruli, double basement membranes between endothelial cells and podocytes were present and, in certain areas, appeared to be fusing. Second, in maturing stage glomeruli, elaborate loops and outpockets of basement membrane projected into epithelial, but not endothelial, sides of capillary walls. When Lowicryl thin sections from newborn rat kidneys were sequentially labeled with rabbit anti-laminin IgG and anti-rabbit IgG-colloidal gold, gold bound across the full width of all GBMs, including double basement membranes and outpockets. The same distribution was obtained when sections from rats that received intravenous injections of rabbit anti-laminin IgG 1 h before fixation were labeled directly with anti-rabbit IgG-colloidal gold. When kidneys were fixed 4 d after anti-laminin IgG injection, however, loops beneath the podocytes in maturing glomeruli were usually unlabeled and lengths of unlabeled GBM were interspersed with labeled lengths. In additional experiments, rabbit anti-laminin IgG was intravenously injected into newborn rats and, 4–14 d later, rats were re-injected with sheep anti-laminin IgG. Sections were then doubly labeled with anti-rabbit and anti-sheep IgG coupled to 10 and 5 nm colloidal gold, respectively. Sheep IgG occurred alone in outpockets of maturing glomeruli and also in lengths of GBM flanked by lengths containing rabbit IgG. These results indicate that, after fusion of double basement membranes, new segments of GBM appear beneath developing podocytes and are subsequently spliced into existing GBM. This splicing provides the additional GBM necessary for expanding glomerular capillaries.

The renal glomerular basement membrane (GBM) is approximately twice the thickness of most other basement membranes, measuring ~150 nm in rats. One reason for its increased thickness is that the GBM originates during development from the apparent fusion or union of two basement membranes: one beneath the vascular endothelium and one beneath the overlying podocytes or visceral epithelium of Bowman's capsule. This fusion is probably physiologically significant, since, in addition to creating a thicker matrix, it results in a double layer of anionic sites in the lamina rara interna and externa that is important in establishing a charge barrier to serum proteins. The fusion of dual basement membranes generally takes place as glomerular capillary loop diameters are increasing, but the mechanism for the assembly of additional GBM in these expanding capillaries has not been examined in detail.

Previous immunofluorescent labeling studies have shown that laminin (10), type IV collagen, and heparan sulfate proteoglycans (21) are all present within developing GBMs. In earlier immunoelectron microscopy experiments, we intravenously injected anti-laminin IgG coupled directly to horseradish peroxidase (HRP) into newborn rats. This labeled basement membranes of glomeruli in all stages of development, from embryonic in the outer renal cortex to almost mature in the inner cortex. In addition, we identified, in maturing stage glomeruli in the inner cortex, extensive loops of apparently newly synthesized basement membrane material located between and beneath developing foot processes of podocytes. These loops were not induced in immature rats by the intravenous injection of anti-laminin IgG because identical structures were also identified within glomeruli of normal, uninjected newborns. These loops are normally not seen in adult glomeruli, however. In the studies presented here, we have used techniques for labeling developing GBM in vivo for postembedding immunogold elec-
Materials and Methods

Proteins and Reagents
Laminin was purified from the murine Englebreth-Holm-Swarm sarcoma by salt extraction, ion exchange, and gel filtration chromatography (1, 27), and characterized as previously described (1). Rabbit and sheep anti-laminin IgGs were purified from sera collected from immunized animals, affinity-isolated from columns of laminin-Sepharose, and shown to be highly specific for laminin (1-3). Laminin-adsorbed rabbit and sheep IgGs and commercially prepared IgGs (Cooper Biomedical, Inc., Malvern, PA) were used in control experiments. Anti-laminin and control IgGs were conjugated directly to activated HRP (type VI, Sigma Chemical Co., St. Louis, MO) as before (1-3). Goat anti-rabbit IgG coupled to 10-nm diameter colloidal gold was obtained from Jackson Immunoresearch Laboratories, Inc., West Grove, PA. Rabbit anti-sheep IgG coupled to 5-nm colloidal gold was purchased from Janssen Pharmaceutica (Beerse, Belgium). Rabbit anti-sheep IgG coupled to 5 nm colloidal gold was purchased from E. Y. Laboratories, Inc., San Mateo, CA.

Experimental Procedures
Newborn Sprague-Dawley rats (Southern Animal Farms, Prattville, AL) were anesthetized with ether 2 d after birth. They then received intravenous injections of 0.3 ml of affinity-purified rabbit anti-laminin IgG, rabbit anti-laminin IgG-HRP, or control IgGs (1.0 mg IgG/ml) via the saphenous vein. For double labeling experiments, rats were re-anesthetized 4-14 d later, and these animals then received intravenous injections of 0.4-0.5 ml affinity-purified sheep anti-laminin IgG.

Tissue Processing
The left kidneys from anesthetized rats were clamped at the hilus and the appropriate fixative solution was simultaneously injected into the cortices. Kidney tissues from newborns that had not received IgG injections were fixed in 1% glutaraldehyde and 1% tannic acid in 0.1 M phosphate buffer, pH 7.3, for 2 h, postfixed in 2% osmium to inactivate residual aldehydes, and treated for an additional 1 h with 0.1% BSA in PBS to minimize nonspecific labeling. Sections from each of two uninjected rats were then incubated sequentially in drops of 15 μg/ml affinity-purified rabbit anti-laminin IgG, or control IgG, and colloidal gold anti-rabbit IgG using protocols described previously (4). Lowicryl sections, 70-nm thick, were picked up on uncoated 400-mesh nickel grids, treated for 1 h with 0.5 M ammonium chloride to inactivate residual aldehydes, and treated for an additional 1 h with 0.1% BSA in PBS to minimize nonspecific labeling. Sections from each of two uninjected rats were then incubated sequentially in drops of 15 μg/ml affinity-purified rabbit anti-laminin IgG, or control IgG, and colloidal gold anti-rabbit IgG using protocols described previously (4). Lowicryl sections from six rats that received intravenous injections of rabbit anti-laminin IgG, or control IgG (two rats), were incubated directly with anti-rabbit IgG-colloidal gold for 64 h at 4°C in sealed microfuge tubes. Incubations for shorter periods generally resulted in significantly less immunogold labeling with this technique. Sections from five rats that received dual injections of rabbit and sheep anti-laminin IgG, or control IgGs (one rat), were labeled first with rabbit IgG-colloidal gold (80 nm) for 64 h, washed in buffered albumin, and then treated for 24 h with 0.5% normal rabbit serum in buffered albumin. Grids were then re-labeled with anti-sheep IgG-colloidal gold (5 nm) for 64 h. All grids labeled with immunogold were thoroughly washed with buffered albumin and then dried. Tissue sections were then stained for 30 s with 2% uranyl acetate and for 1 min with lead citrate. Electron microscopy was conducted with an accelerating voltage of 60 kV. Lengths of GBM from photographic prints were measured on a digitizing tablet using a computer morphometric program (R & M Biometrics, Inc., Nashville, TN) (2). Gold particle counts were expressed as number of colloidal gold particles per micrometer linear length of GBM.

Results
The two developmentally and morphologically distinct stages of GBM assembly were clearly observed with the ad-
Figure 2. Capillary loop from maturing stage glomerulus of normal newborn rat kidney. Numerous irregular loops and outpockets of basement membrane (arrows) are present beneath differentiating foot processes of podocytes (Ep). Note that electron-dense and electron-lucent layers within these outpockets can be distinguished. Outpockets do not extend into the endothelial cell layer (En). In contrast to outpockets, double basement membranes are generally not observed in maturing stage glomeruli. CL, capillary lumen; US, urinary space. Bar, 1.0 μm.

A solution of tannic acid to aldehyde fixatives (Figs. 1 and 2). First, in early (S-shaped and developing capillary loop stage) glomeruli, the two laminae densae of double basement membranes between developing endothelial cells and podocytes were readily identified (Fig. 1). In some cases, the dual laminae densae were only narrowly separated and in these areas may have been in the process of fusing (Fig. 1). In later, maturing stage glomeruli, elaborate, irregular outpockets and loops of basement membrane projecting into the epithelial sides of capillary walls were often present (Fig. 2). These loops contained electron-dense and electron-lucent layers, probably corresponding to the lamina densa and lamina rara, and were otherwise ultrastructurally identical to mature GBM. Basement membrane outpockets were seen most frequently between and beneath podocyte foot processes undergoing development and interdigitation (Fig. 2). Double basement membranes were rarely seen in glomeruli in these stages.

Postfixation, Postembedding Colloidal Gold Immunolabeling of Laminin

When Lowicryl thin sections of formaldehyde-fixed newborn rat kidneys were sequentially labeled with rabbit anti-laminin IgG and anti-rabbit IgG–colloidal gold, gold particles bound to sections specifically overlying basement membranes (Fig. 3, a–c). In developing glomeruli at early stages, gold bound to both laminae densae of double basement membranes as well as to the lamina rara interna beneath the endothelium and the lamina rara externa beneath the epithelium (Fig. 3 a). There was little or no gold binding to the...
central region between the two basement membranes when the two laminae densae were widely separated, however (Fig. 3 a). In glomeruli at later developmental stages, the full width of the GBM was labeled (Fig. 3, b and c). In addition, gold bound to intracellular vesicular structures within the glomerular epithelium (Fig. 3 b). The basement membrane outpockets found in maturing glomeruli were labeled with anti-laminin IgG throughout their full dimensions (Fig. 3 c). Gold was also present in the lamina rara interna, lamina densa, and lamina rara externa of the GBM, but appeared to occur most frequently in the lamina densa (Fig. 3 c). Anti-rabbit IgG-colloidal gold did not bind to Lowicryl sections treated with control, laminin-adsorbed rabbit IgG (Fig. 4).

In Vivo Labeling of Laminin Detected by Postembedding Colloidal Gold Techniques

To determine the GBM distribution of anti-laminin IgG introduced in vivo, Lowicryl sections from newborn rats that had received intravenous injections of rabbit anti-laminin IgG 1 h before fixation were treated directly with anti-rabbit IgG-colloidal gold. As shown in Fig. 5 a, gold bound exclusively and in high densities to the developing GBM. Intravenously injected anti-laminin IgG was also present throughout the full extent of the basement membrane outpockets characteristic of maturing glomeruli (Fig. 5 a) as seen previously when anti-laminin IgG was applied to fixed tissue (Fig. 3 c). To evaluate whether the distribution of GBM-bound anti-laminin IgG changed during GBM assembly, rat kidneys were fixed and processed for postembedding immunolabeling 4, 5, and 14 d after IgG injection. Capillary loops in maturing stage glomeruli were then photographed and the numbers of colloidal gold particles bound to the GBM were counted and compared with what was seen 1 h after injection. In general, a marked reduction in colloidal gold binding to Lowicryl sections was seen at 4 d and, in particular, the
GBM outpockets were often completely unlabeled (Fig. 5 b). Even less gold bound to sections from kidneys fixed 14 d after anti-laminin IgG injection (Fig. 5 c). However, gold generally was not restricted to any one layer of the GBM and instead was grouped in small clusters across the full width. In addition, there were unlabeled lengths of GBM interspersed between labeled lengths (Fig. 5 c). Anti-rabbit IgG–colloidal gold did not bind to Lowicryl sections from control, un.injected rats, or rats that received injections of laminin-adsorbed rabbit IgG or commercial rabbit IgG.

**Anti-Laminin IgG–HRP Experiments**

Rats that had received intravenous injections of sheep anti–laminin IgG coupled directly to HRP 1 h before fixation had peroxidase reaction product throughout all of their GBMs, as well as in the outpockets, in apparently uniform densities, as shown before (2). When kidneys were fixed 4–6 d after anti-laminin IgG–HRP injection, however, GBMs in maturing stage glomeruli usually showed highly variable HRP reaction product (Fig. 6). Here, HRP labeling within capillary loops was often interrupted with weakly labeled and unlabeled lengths of GBM (Fig. 6). When present, however, HRP reaction product appeared to extend across the full thickness of GBM except in areas underlying outpockets. In these cases, the outpockets were usually unlabeled with anti-laminin IgG, as previously shown with immunoperoxidase (2), and here with postembedding immunogold labeling techniques (Fig. 5 b).

**Double Labeling Experiments**

Lowicryl sections from rats that had received intravenous injections of rabbit anti–laminin IgG and sheep anti–laminin IgG at separate times of development were doubly labeled with anti–rabbit IgG and anti–sheep IgG coupled to 10 nm and 5 nm diameter colloidal gold, respectively. In these cases, the total amount of either colloidal gold binding to sections was the same as when sections were labeled singly (data reviewed but not shown). In doubly labeled sections from dually injected rats, the distribution patterns of rabbit and sheep anti–laminin IgG in maturing stage glomeruli were frequently different, however. In rats that received sheep anti–laminin IgG 4–14 d after rabbit anti–laminin IgG, sheep, but not rabbit IgG, was often present within GBM outpockets, whereas both IgGs were present together in GBM immediately beneath the outpockets (Fig. 7, a and b). In addition, in areas of apparently completely assembled GBM, sheep anti–laminin IgG was also present alone in lengths that were flanked by lengths containing rabbit anti–laminin IgG (Fig. 7 c).

**Discussion**

The purpose of these experiments was to further study the process of GBM assembly in developing kidneys. Our observations indicate that in maturing stage glomeruli, newly synthesized matrix, which is probably derived mainly from the podocytes, is spliced into existing GBM.

**Laminin Distribution within Developing GBMs**

The results from this study with immunogold labeling confirmed earlier experiments with immunoperoxidase (2), showing that anti–laminin IgG bound to the full thickness of each of the double basement membranes found in early stage glomeruli. Since this double basement membrane subsequently appears to fuse, the mature GBM therefore probably contains at least a double layer of laminin. That both the endothelium and epithelium contribute to GBM formation has been previously shown in other studies using anti-type IV collagen antibodies (25). In later developmental stages, however, double basement membranes were generally not present and anti–laminin IgG bound throughout the full width of the GBM between the endothelium and epithelium. Postembedding colloidal gold immunolabeling of laminin in adult kidneys has similarly shown laminin throughout the full width of the mature GBM (4). In addition, laminin was immunolocalized in newborn kidneys throughout the full extent of the irregular loops of basement membrane material that projected into the epithelium of maturing stage glomeruli. Since these loops and outpockets ultrastructurally resemble basement membranes, contain electron-dense and electron-lucent layers corresponding to the lamina densa and lamina rara, and contain anionic sites in the electron-lucent layers as shown by labeling with cationized ferritin (5), we believe that they probably represent at least a precursor form of genuine GBM. Previous experiments suggested that these basement membrane loops were synthesized mainly by the epithelium (2). The immunogold labeling of laminin intracellularly within maturing podocytes, but not the endothelium in maturing glomeruli, therefore supports these earlier observations.

**Postembedding Localization of Intravenously Injected Anti–Laminin IgG**

Techniques were developed here for labeling developing basement membranes in vivo with the intravenous injection of anti–laminin IgG, and then detecting this GBM-bound IgG with postembedding colloidal gold immunolabeling procedures. In kidneys from animals that had received injections 1 h before fixation, gold was abundantly present throughout the full width of GBM as well as in the outpockets, as seen.
when thin sections from uninjected rats were sequentially processed for routine postembedding immunolabeling. In other words, anti-laminin IgG, whether applied in vivo under presumably normal filtration conditions, or in vitro to fixed, embedded kidney sections, bound in precisely the same distribution patterns to the GBM. When Lowicryl sections from rats that had received injected anti-laminin IgG 4 d previously were labeled, however, significantly less gold bound to the GBM. In addition, the GBM outpockets at this time were usually entirely unlabeled with colloidal gold. This result indicates (a) that the unlabeled outpockets were assembled after IgG injection, and (b) that anti-laminin IgG, once bound to the GBM in vivo, did not redistribute in discernable amounts to newly synthesized laminin. However, even less anti-rabbit-colloidal gold bound to Lowicryl sections from rats that had received rabbit anti-laminin IgG 14 d before fixation. We do not believe that this progressive reduction in immunogold binding was due to significant levels of in vivo dissociation of anti-laminin IgG from the GBM, however, for several reasons. First, all of the antibodies used in this study were affinity-isolated and, consequently, had been selected for their ability to combine relatively tightly with laminin. Second, quantitative immunofluorescence microscopy of mature rat glomeruli labeled in vivo with anti-laminin IgG has previously shown that most of the bound IgG remains stably associated with the GBM for at least several weeks (3). Immunofluorescence microscopy of newborn rat kidneys has also shown persistent binding of anti-laminin IgG to developing GBM (2). Finally, when kidneys labeled in vivo with anti-laminin IgG-HRP 4-6 d before fixation were examined, HRP reaction product in capillary loop GBM of maturing stage glomeruli was, in areas, nonlinear and was punctuated with numerous unlabeled sections of GBM. The possibility that anti-laminin IgG-HRP completely dissociated from some lengths of GBM, but not others, therefore seems unlikely. Nevertheless, patterns of
bound IgG clearly changed quantitatively with subsequent development. As discussed below, we believe that this primarily reflects the addition of newly synthesized basement membrane into expanding glomerular walls.

In further experiments, newborn rats that had received rabbit anti-laminin IgG were re-injected 4–14 d later with sheep anti-laminin IgG and the different IgGs were then localized in Lowicryl sections by postembedding double immunolabeling with the appropriate gold conjugates of distinguishable sizes. The results showed that sheep IgG often occurred alone within the GBM outpockets and in lengths of apparently mature GBM that were usually flanked on either side with stretches of GBM containing rabbit IgG. Taken together, we believe that the best interpretation of these findings is that in maturing stage glomeruli, new segments or patches of basement membrane, which first appear by transmission electron microscopy as loops and outpockets beneath the podocytes, are spliced into existing GBM. This process would result in the dilution of old GBM with new, and, therefore, would explain the overall reduction in immunogold binding to sections from newborn rats fixed several days after in vivo GBM labeling. The interrupted pat-

Figure 7. Lowicryl sections from rats that received rabbit anti-laminin IgG at 2 d of age and were then re-injected with sheep anti-laminin IgG at 6 (a and b) or 16 d of age (c). Kidneys were fixed 1 h after sheep IgG injection. Sections were doubly labeled with anti-rabbit IgG-10 nm colloidal gold and anti-sheep IgG-5 nm colloidal gold. In a and b, only sheep anti-laminin IgG is present within the outpockets (arrowheads) whereas both sheep and rabbit IgG (arrows) are present together in subjacent GBM. In c, there are lengths of GBM that contain only sheep IgG (brackets) interspersed with segments containing rabbit IgG (arrows). (cf. Fig. 5 c). En, endothelium; Ep, epithelium; US, urinary space. Bars, 0.2 μm.
surface area can be achieved by the progressive splicing of new patches of basement membrane into existing GBM. Splicing of newly synthesized basement membrane into existing matrix may not be restricted to developing renal glomeruli, however, and may take place frequently in other sites during development as well as during wound healing.

Once the glomerulus has fully blossomed, widespread splicing of new basement membrane patches into the GBM is no longer necessary and extensive basement membrane outpockets beneath the epithelial podocytes are normally not observed in mature kidneys. However, numerous subepithelial knobs or spikes of basement membrane, which are ultrastructurally similar to the outpockets seen here in immature rats, have recently been reported in adult rats and mice with different forms of experimental anti-GBM glomerulonephritis (18-20). In addition, subepithelial knobs are a relatively common structural feature of several human nephropathies. This may be due, among other possibilities, to a resumption of high levels of basement membrane biosynthesis by podocytes in response to injury.

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