Integrin alfa3beta1 and tetraspanin CD151 in particular in the skin and the kidney
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Glomerular Extracellular Matrix Components and Integrins.
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Glomerular Extracellular Matrix Components and Integrins

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It has become apparent that extracellular matrix components and their cellular receptors, the integrins, are important regulators of glomerular development and function. In this rapidly evolving field we studied the production of extracellular matrix components and integrins by rat glomerular visceral epithelial and mesangial cells, using molecular probes and antibodies that have recently become available. Special attention was paid to laminin isoforms and to splice variants of the integrin subunits α3 and α6. Results were compared to the in vivo expression in human fetal, newborn and adult kidneys.

The mesangial cells were found to produce laminin-1, nidogen and two as yet unidentified laminin isoforms with putative α chains of about 395 (αx) and of 375 kDa (αy), tentatively described before as bovine kidney laminin. Furthermore, they expressed the integrins α1β1, α2β1, α3Aβ1, α5β1, αvβ3, αvβ5, and small amounts of α6Aβ1 and α6Bβ1. The glomerular visceral epithelial cells produced the two new laminin isoforms mentioned above, laminin-5, but no laminin-1 or nidogen. The integrins α2β1, α3Aβ1, α6Aβ4, α6Bβ4 and the integrin subunit αv were found to be expressed.

We show that during nephrogenesis, the laminin α1 chain disappears and is replaced by another α chain, possibly one of the two as yet unidentified α chains mentioned above. The laminin β1 chain is replaced by the β2 chain somewhat later in glomerular development. In general, the integrins found to be expressed in glomeruli of adult kidney were consistent with those found in cultured glomerular visceral epithelial and mesangial cells. No splice variant switch of the integrin α3 or α6 subunits could be demonstrated during nephrogenesis.

Our results suggest an important role for the mesangial cell in providing nidogen as a crucial component of the supramolecular structure of the glomerular basement membrane. Furthermore our results indicate that laminin α1β1γ1 and α1β2γ1 isoforms are important in the glomerulus of adult kidney and that the integrin α3Aβ1 is the main integrin receptor for laminin isoforms on glomerular visceral epithelial and mesangial cells, both in vitro and in vivo.

Keywords: Laminin, extracellular matrix, integrins, kidney, development

Classification Categories: ABBREVIATIONS
ECM, Extracellular Matrix, GVEC, Glomerular Visceral Epithelial Cell, GBM, Glomerular Basement Membrane
INTRODUCTION

The glomerular basement membrane (GBM) is a specialized extracellular matrix (ECM) network, functioning as a size and charge selective filter barrier between the circulation and the urinary space. Furthermore it has a role in cell attachment, growth and development. It is composed of collagen IV, nidogen, laminins, fibronectin and heparan sulfate proteoglycans (Weber, 1992). Fibronectin is partly derived from the circulation, while the other components are produced in situ. It is believed that the GBM is deposited at the interface of the visceral epithelium and the endothelium as two distinct layers, early in nephrogenesis. During development, they fuse in the middle (Sariola et al., 1984). Which cell type is responsible for the production of the different GBM components is only partly known (Ekblom, 1981; Abrahamson and St.John, 1994). The podocyte is thought to play a role in maintaining the barrier function, the contractile mesangial cell embedded in the matrix can vary the capillary diameter and thereby the glomerular filtration rate (Kanwar and Rosenzweig, 1982; Kashgarian and Sterzel, 1992).

One of the first components expressed in the primitive GBM, appears to be laminin-1. Like the other laminin isoforms (see below), laminin-1 is a heterotrimer. It consists of an α1, β1 and γ1 chain of which the production during nephrogenesis is not fully synchronized. First, the laminin β1 and γ1 chains are synthesized by mesenchymal cells. During induction of the blastema, after which cell polarization takes place, the α1 chain is transiently produced. In kidney organ cultures, the production of the α1 chain is largely confined to the epithelium and seems to be important in tubulogenesis, which succeeds glomerulogenesis (Ekblom et al., 1990).

In addition to laminin-1, six other laminin isoforms have been described. All these isoforms are composed of an α, β and γ chain. Five α, three β and two γ chains have been identified. These chains occur in different combination in the various laminin isoforms (Burgeson et al., 1994; Delwel and Sonnenberg, 1996). The β2 chain, shown to be part of laminin-3 and laminin-4, was detected in the GBM (Noakes et al., 1995). Probably, still other laminin isoforms occur in the kidney. In fact, in bovine kidney an alternative laminin α chain has already been found, and in human and murine kidney transcripts of two other laminin α chains, α4 and α5, respectively, have been described (Lindblom et al., 1994; Livaininen et al., 1995; Miner et al., 1995).

Integrins are involved in the attachment of glomerular cells to the GBM (Adler et al., 1992; Baraldi et al., 1994; Cybulsky et al., 1992; Korhonen et al., 1990, 1992). Integrins are heterodimeric transmembrane receptors consisting of an α- and β-subunit. Various subunit combinations form at least 20 different integrins known at present. Their expression is cell type specific and their ligand specificities overlap considerably (Hynes, 1992; Sonnenberg, 1993). Via adhesion and signal transduction they influence many fundamental biological processes such as embryogenesis, wound healing and the maintenance of tissue integrity (Schwartz et al., 1995; Clark and Brugge, 1995; Yamada and Miyamoto, 1995). Due to alternative splicing of mRNA, variants of the cytoplasmic domain of some α- and β-subunits are produced (Tamura et al., 1991; Hogervorst et al., 1991; Song et al., 1993; Collo et al., 1993; Ziober et al., 1993; van der Flier et al., 1995; Zidkova et al., 1995). Since in some cell types splicing of the mRNA for these subunits is developmentally regulated, these variants may influence cell function (Cooper et al., 1991; Hierck et al., 1993; Ziober et al., 1993; Collo et al., 1993; van der Flier et al., 1995; Zidkova et al., 1995).

As shown by others, many different integrins are present in the glomerulus. However, there are some discrepancies in results which may be due to the antibodies and/or the methods applied (Korhonen et al., 1990, 1992; Patey et al., 1994). Of the laminin receptors, the α3β1 integrin has been shown to be expressed glomerular by epithelial cells, mesangial cells and endothelial cells in vivo and in cultured cells, respectively, and α6β1 is expressed during cell polarization in glomerulogenesis (Sorokin et al., 1990). Two cytoplasmic splice variants of the α3 and α6 subunits, A and B, have been described (Hogervorst et al., 1991; Tamura et al., 1991). Although α3A
is expressed in the adult glomerulus and \( \alpha 6 \beta \) in tubuli, nothing is known about the expression of these splice variants during kidney development.

In this study, we made an inventory of ECM components and integrins in cultured rat glomerular visceral epithelial (GVEC) and mesangial cells and compared our results to their presence \textit{in vivo} in normal adult and fetal kidney, concentrating on laminin isoforms and their integrin receptors. Additionally, we have studied the expression of the cytoplasmic variants of the integrin \( \alpha 3 \) and \( \alpha 6 \) subunits, using recently obtained monoclonal antibodies. Results on the distribution of ECM components are discussed in the light of current concepts of basement membrane assembly and its three dimensional composition.

**MATERIALS AND METHODS**

**Cell Culture and Antibodies**

Mesangial cell cultures were obtained from established cell lines from Wistar rats (Floge et al., 1993). Passage numbers 10 to 40 were used. Cells were maintained in DMEM containing 1000 mg/l glucose, supplemented with 100 U/ml penicillin, 100 \( \mu \)g/ml streptomycin, 5 \( \mu \)g/ml bovine insulin (Sigma Chemical Co.) and 10% (v/v) fetal calf serum (FCS) (Gibco-BRL, Paisley, UK).

GVEC, an established cell line derived from explants of glomerular podocytes from Sprague-Dawley rats, has been characterized and described previously (Quigg et al., 1988). The cells were grown in tissue culture flasks on a collagen gel (Vitrogen 100, Collagen Corp., Ca). The medium was composed of 45% (v/v) DMEM (Gibco-BRL), 45% (v/v) HAM-F10 (Gibco-BRL) containing K1 hormone mix (5 \( \mu \)g/ml insulin, 25 \( \mu \)g/ml prostaglandin E1, 5 \( \times \) \( 10^{-8} \) M triiodothyronine, 1 \( \times \) \( 10^{-8} \) M \( \text{Na}_2\text{SeO}_3 \), 5 \( \mu \)g/ml transferrin, 5 \( \times \) \( 10^{-8} \) M hydrocortisone, all purchased from Sigma Chemical Co. (St.Louis, MO), 100 U/ml penicillin (Gibco-BRL), 100 \( \mu \)g/ml streptomycin (Gibco-BRL) and 5% (v/v) NuSerum (Collaborative Research Inc., Bedford, ME). The cells were passaged using 0.2% collagenase type IA (330 U/mg (Sigma Chemical Co.) in HBBS (Gibco-BRL), followed by two washing steps with HBBS. All experiments were performed with passage numbers 38-45.

Rabbit polyclonal antibodies were kindly provided by the following investigators: by Dr. R. Timpl anti-laminin-1 (\( \alpha 1 \beta 1 \gamma 1 \)) (E8#970) (Paulsson et al., 1985), anti-laminin \( \alpha 1 \) chain (E3#992) (Paulsson et al., 1985) and anti-nidogen (Fox et al., 1991) by Dr.E. Envall, anti-laminin \( \alpha 2 \) chain (MS#1381) (Leivo and Engvall, 1988) and collagen IV (Engvall et al., 1982), by Dr. R.E. Burgeson anti-laminin-5 (\( \alpha 3 \beta 3 \gamma 2 \)) (Marinkovich et al., 1992), by Dr. G. Tarone, anti-integrin subunits \( \alpha 1 \), \( \alpha 4 \), \( \alpha 5 \), \( \alpha v \) and \( \beta 1 \) (Defilippi et al., 1991), by Dr. R.H. Kramer, anti-\( \alpha 7 \beta \), by Dr. L.F. Reichardt anti-\( \alpha 8 \) (Bossey et al., 1991) and by Dr. H. Kemperman anti-\( \beta 4 \) (Niessen et al., 1994), \( \beta 5 \) and \( \beta 6 \) (Sánchez-Aparicio et al., 1997). Antibodies against fibronectin were obtained from Telios Pharmaceuticals, Inc. (San Diego, CA). The monoclonal antibodies used are listed in Table 1.

**Cell Labeling and Immunoprecipitation**

For metabolic labeling, cells were grown to 80% confluency and incubated overnight with 90% methionine-free minimal essential medium (Gibco-BRL), 10% DMEM and 50 \( \mu \)Ci/ml \( [\text{15S}] \)-methionine (1250 Ci/mmol; Amersham Corp., Amersham, UK). Culture medium was then collected and cleared of cell debris by centrifugation in an Eppendorf centrifuge at 14,000 rpm at 4\( ^\circ \)C. Detergent (1% v/v Nonidet P40) and protease inhibitors (10 \( \mu \)g/ml soybean trypsin inhibitor, 10 \( \mu \)g/ml leupeptin, 1 mM phenylmethylsulfonyl fluoride) were added. Culture medium was preclarified with protein A-Sepharose CL-4B beads (Pharmacia LKB Biotechnology Inc., Uppsala, Sweden). Samples of the preclarified medium were immunoprecipitated at 4\( ^\circ \)C, with antibodies previously bound to protein A-Sepharose CL-4B beads (Pharmacia LKB Biotechnology Inc., Uppsala, Sweden). Samples of the preclarified medium were immunoprecipitated at 4\( ^\circ \)C, with antibodies previously bound to protein A-Sepharose beads, or to protein A-Sepharose beads coated with rabbit antimouse or rabbit anti-rat IgG at room temperature.

For surface labeling, cells were detached as described above, washed three times with PBS and labeled with \( ^{125}\text{I} \) by the lactoperoxidase/hydrogen.
peroxidase method as described previously (Sonnenberg et al., 1986a). Cells were lysed for 30 min at 4°C in lysis buffer, containing 1% (v/v) Nonidet P40, 100 mM NaCl, 25 mM Tris-HCl, pH 7.5 and protease inhibitors. Clarification of lysates was followed by incubation for 1 h with antibodies as mentioned above.

The beads carrying the immunocomplexes were washed and treated with SDS sample buffer. Immunoprecipitates of matrix components and integrins were analyzed by SDS-PAGE under reducing or non-reducing conditions. Reduction was effectuated by adding 5% β-mercaptoethanol.

**Immunoperoxidase Staining of Tissue Sections**

For immunoperoxidase staining, 3 μm thick cryostat sections of human kidney from a fetus, newborn or an adult in which all glomerular developmental stadia are present, were used. A two-step indirect procedure with horseradish peroxidase conjugated second antibodies, visualized by hydrogen peroxide and 3,3′-diaminobenzidine tetrachloride or 3′,3′-amino-9-ethylcarbazole (Sigma Chemical Co.) as substrate, was used. The sections were counterstained with hematoxylin and mounted in Depex (British Drug House Chemicals, Poole, UK). Control stainings were performed in the absence of a primary antibody.

**Northern Blot Analysis**

Total RNA from GVEC and mesangial cells was isolated by the urea/lithium chloride method (Barlow et al., 1963). RNA samples (10 μg of each cell line) were separated by electrophoresis on a 1% agarose gel containing formaldehyde and transferred to nitrocellulose. Hybridizations were carried out for 16 h at 42°C with cDNA probes that had been 32P-labeled by random priming. The following probes were used: a 2.7-kb fragment of the murine laminin β1 chain cDNA (LamB1) and a 1.3-kb fragment of murine laminin γ1 chain cDNA (LamC1) from Dr. R. Deutzmann (Deutzmann et al., 1985), a 2.5-kb fragment of rat laminin β2 chain cDNA (LamB2) from Dr. J. Sanes (Hunter et al., 1989), and a 2.0-kb fragment of murine laminin γ2 chain cDNA (LamC2) from Dr. G. Meneguzzi (Galliano et al., 1995). For the detection of laminin-5, RNA isolated from RAC-11P and RAC-5E cells was used as a positive and negative control, respectively (Sonnenberg et al., 1986b). As a control for the amounts of RNA loaded on to the gel, the same RNA blot was hybridized with a 1.2-kb fragment cDNA probe for
human glyceraldehydephosphatedehydrogenase (GAPDH).

RESULTS

Production of ECM Components In Vitro

ECM proteins produced by GVEC and mesangial cells were immunoprecipitated from the culture media of $[^{35}S]$-metabolically labeled cells, using antibodies against various laminin isoforms, nidogen, collagen IV or fibronectin. Results are shown in Fig 1. Polyclonal anti-laminin-1 ($\alpha/\beta_1\gamma_1$) (lane 1) immunoprecipitated six polypeptides of 400, 395, 375, 220, 210 and 150 kDa from the medium of mesangial cells. The same pattern of bands was obtained with the laminin $\gamma_1$ chain specific antibody (lane 6) and with anti-nidogen (lane 3), while antibodies against $\alpha_1$ only recognized the 400, 220, 210 and 150 kDa polypeptides (lane 2). The size of the 400 and 150 kDa polypeptides closely corresponds to that of the laminin $\alpha_1$ chain and of nidogen. The 220 and 200 polypeptides probably represent the laminin $\beta_1$ and $\gamma_1$ chains, respectively. Expression of $\beta_1$ and $\gamma_1$ mRNAs by mesangial cells was detected by Northern blot analysis (Fig 2, lane 3). The 395 and 375 kDa polypeptides that could not be precipitated with $\alpha_1$ specific antibodies (lane 2), probably are two, as yet undetected new laminin $\alpha$ chains, provisionally called $\alpha_x$ and $\gamma_y$. Laminin $\alpha_2$ chain (lane 4), $\beta_2$ chain (lane 5) or laminin-5 (lane 7) were not, but fibronectin and collagen IV were detected as shown in lanes 8 and 9, respectively.

Immunoprecipitations performed on the conditioned medium of GVEC with the polyclonal antisem against laminin-1 revealed only four polypeptides of 395, 375, 220 and 210 kDa (lane 1). Again the 220 and 210 kDa polypeptides represent the laminin $\beta_1$ and $\gamma_1$ chains. Because only small amounts of the laminin $\gamma_1$ chain were produced, it was difficult to detect this chain by immunoprecipitation (lanes 1 and 6), but the mRNA encoding the laminin $\gamma_1$ chain as well as that for the $\beta_1$ chain could easily be detected by Northern blot analysis (Fig 2, lane 4). In contrast to

![FIGURE 1](image-url) Analysis of $[^{35}S]$-methionine labeled ECM components in conditioned medium of MC (left) and GVEC (right) by immunoprecipitation using antibodies against laminin-1 (lane 1), laminin $\alpha_1$ chain (lane 2), nidogen (lane 3), laminin $\alpha_2$ chain (lane 4), laminin $\beta_2$ chain (lane 5), laminin $\gamma_1$ chain (lane 6), laminin-5 (lane 7), fibronectin (lane 8) and collagen IV (lane 9). Samples were analyzed by SDS-PAGE on a 5% reduced gel. The positions of the various laminin chains are indicated in the left margin.
mesangial cells, GVECs produced no laminin-1, as is shown by the absence of the 400 kDa band in the immunoprecipitates with anti-laminin 1 (lane 1) and anti-laminin 31 (lane 2) antisera. Furthermore, no nidogen was precipitated with nidogen specific anti-

 bodies (lane 3). As by mesangial cells, two laminin α chains of 395 (αx) and 375 kDa (αy) were produced by GVEC (lane 1). No laminin α2 (lane 4) and β2 chains (lane 5), but laminin-5 (α3β3γ2) (lane 7), fibronectin (lane 8) and collagen IV (lane 9) were detected. Northern blot analysis (Fig 2) confirmed the expression of the genes coding for the laminin β3 and γ2 chains, both present in laminin-5, in GVEC but not in mesangial cells (lane 4). As a positive control for laminin-5 production, the RAC-11P cell line was used (lane 2) and a non laminin-5 producing cell line, RAC-5E, as a negative control (lane 1).

Expression of Integrin Subunits in vitro

The expression of integrins was studied by immunoprecipitation experiments using 125I surface labeled cells and a panel of monoclonal antibodies against different cytoplasmic and extracellular domains, and polyclonal antisera against cytoplasmic domains of integrin subunits (Fig 3, Table 3). From the lysates of mesangial cells, α1β1, α2β1, α3β1, α5β1, α6β3 and α6β5 were precipitated as well as very small amounts of α6β1 and α6β1 (only visible after prolonged exposure). No α3B, α4, α7B, α8 and β4 were detected. From the lysates of GVEC (Fig 3, Table 3), α1 and α3A were precipitated in association with β1, and α6A and α6B in association with β4. Polyclonal antibodies precipitated αv and an associated β subunit of about 95 kDa. This β subunit is not β1, β3 or β5, because antibodies against these subunits did not coprecipitate αv. The size of 95 kDa suggests that it might be β8. No α1, α3B, α4, α5, α7B and α8 were detected.

Immunohistochemical Staining

The production of ECM components and the expression of integrins in vitro was compared with the in vivo expression in frozen kidney sections from a 16-weeks old human fetus, 1-month old newborn and an adult (Table 2). Antibodies against nidogen and collagen IV reacted with GBM and, to a lesser extent, with the mesangium in glomeruli from the comma-stage onward, while antibodies against fibro-
FIGURE 3  Analysis of integrins on mesangial cells and GVECs. Lysates of \(^{125}\text{I}\)-labeled mesangial cells (left) and GVEC (right) were immunoprecipitated with antibodies against \(\alpha_1\) (lane 1), \(\alpha_2\) (lane 2), \(\alpha_3\)A (lane 3), \(\alpha_3\)B (lane 4), \(\alpha_4\) (lane 5), \(\alpha_5\) (lane 6), \(\alpha_6\)A (lane 7), \(\alpha_6\)B (lane 8), \(\alpha_v\) (lane 9), \(\beta_1\) (lane 10), \(\beta_3\) (lane 11), \(\beta_4\) (lane 12) or \(\beta_5\) (lane 13). Samples were analyzed by SDS-PAGE on a 5% nonreduced gel. Positions of the various integrin subunits are indicated in the left margin.

TABLE 2  Immunohistochemical staining of normal human fetal, newborn and adult kidney.*

<table>
<thead>
<tr>
<th>ECM Antigen</th>
<th>Fetal</th>
<th>Newborn</th>
<th>Adult</th>
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<tr>
<td>(\alpha_1) laminin</td>
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<tr>
<td>(\alpha_2) laminin</td>
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<td>(\beta_1) laminin</td>
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<td>(\beta_2) laminin</td>
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<td>(\gamma_1) laminin</td>
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<tr>
<td>laminin-5</td>
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<td>-</td>
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<tr>
<td>nidogen</td>
<td>-</td>
<td>++</td>
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<tr>
<td>collagen IV</td>
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<td>+++</td>
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<tr>
<td>fibronectin</td>
<td>-</td>
<td>+</td>
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Integrin  | Fetal | Newborn | Adult |
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<tr>
<td>(\alpha_3)A</td>
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<td>+</td>
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<tr>
<td>(\alpha_3)B</td>
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<td>(\alpha_4)</td>
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<td>(\alpha_6)A</td>
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<td>(\alpha_6)B</td>
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<td>(\alpha_7)B</td>
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<td>(\alpha_8)</td>
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<tr>
<td>(\beta_1)</td>
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<td>(\beta_4)</td>
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<td>(\beta_5)</td>
<td>-</td>
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<td>+f</td>
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*Abbreviations are: ECM, extracellular matrix; c-stage, comma-shaped body stage; s-stage, s-shaped body stage; v-stage, vascular stage; GBM, glomerular basement membrane; GVEC, glomerular visceral epithelial cell/podocyte; MM, mesangial matrix; MC, mesangial cells; f, focal staining; (-), no detectable staining; (±), weak or variable staining; (+), clear, consistent staining; (++), strong staining; (+++), very strong staining; (0), not determined.
TABLE 3 Integrin subunit expression: comparison of immunohistochemical staining of fetal and adult kidney with immunoprecipitation data from cell culture supernatants.1

<table>
<thead>
<tr>
<th>Antigen</th>
<th>GVEC</th>
<th>MC</th>
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<tr>
<td></td>
<td>in vivo</td>
<td>in vitro</td>
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<td></td>
<td>Adult</td>
<td>Fetus</td>
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<td>α1</td>
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<td>α3A</td>
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<td>α3B</td>
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<td>α4</td>
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<td>α6A</td>
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<td>α6B</td>
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<td>α7B</td>
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<td>α8</td>
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<td>αV</td>
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<td>β5</td>
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<td>β6</td>
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</table>

1Abbreviations are: GVEC, glomerular visceral epithelial cells; MC, mesangial cells; (-), no detectable staining; (±), weak or focal staining; (+), clear, consistent staining; (++), strong staining; (+++), very strong staining; (0), not determined.

in vivo: immunoperoxidase staining on renal tissue
in vitro: cell culture experiments

nectin produced a more pronounced staining in the mesangium. In fetal glomeruli, the laminin α1 chain was seen in the earlier, more primitive glomerular stages (Fig 4A-B) and it gradually disappeared after the s-stage (Fig 4C). No laminin α2 chain could be detected. Consistent with the results of a previous report (Miner et al., 1995), we found that the β1 chain appeared after cell polarization and disappeared from the vascular stage onward (not shown). In contrast, the laminin β2 chain was absent in the primitive glomerular stages in the kidney of the fetus and the newborn, but it appeared from the vascular stage onward (Fig 5A-C). The γ1 chain was present throughout development, already appearing in the primitive basement membrane. It was still present in adult kidney (Fig 6A-C). Laminin-5 was not detected in the kidney at any developmental stage, nor was it present in adult glomeruli. Laminin-5 was, however, present in the vessel walls of the adult kidney, especially in the glomerular hilus, and in the basement membrane of distal and collecting tubules (Fig 7).

The expression of integrins was investigated with various antibodies against integrin subunits and compared with the expression in vitro (Table 3). Special attention was paid to a possible role of the α3 and α6 splice variants. The α3A splice variant is abundantly and increasingly expressed during development from the s-shaped body onward, appearing at about the same time that the laminin β2 chain appeared and the β1 chain disappeared. In the adult stage it is expressed by both podocytes and mesangial cells and perhaps also by endothelial cells (Fig 8A). There was no reaction with anti-α3B or with anti-α6A antibodies in any of the developmental stages. From the vascular stage onward, α6B was detected only in a focal pattern, probably related to the mesangium. α6B was strongly expressed by tubular epithelial cells (Fig 8B).

The β1 integrin subunit was present in all developmental stages appearing at the comma-stage and remaining present in adult glomeruli on all cell types. The α1 subunit was found in primitive glomerular
FIGURE 4  Distribution of the laminin α1 chain in the developing human kidney of a 16-week old fetus (A), 4-week old newborn (B) and an adult (C). The laminin α1 chain specific antibody reacts with the primitive basement membrane of the tubuli (T) and comma-stage body (C). A weak immunostaining for α1 is present in the s-shaped stage (S) and some residual staining is still present in the vascular stage (V) and adult glomerulus (A). Also the basement membranes of small vessels are stained (v). (See color plate I).

FIGURE 5  Distribution of the laminin β2 chain in the developing human kidney of a 16-week old fetus (A), 4-week old newborn (B) and adult (C). No immunostaining for β2 is present in the more primitive glomerular developmental stages (not shown). There is a weak staining seen in the basement membrane in the vascular stage (V), becoming more profound in the primitive glomerulus (P) of the newborn and in the adult glomerulus (A). T = tubuli, v = vessels. (See color plate II).
stages and expression increased during development. In adult glomeruli it was expressed by the mesangial cells. There was weak focal staining for \( \alpha 2 \) and \( \alpha 5 \) in the adult glomerulus and \( \alpha 2 \) was probably expressed by both the GVEC and mesangial cells and \( \alpha 5 \) only by mesangial cells. Similarly, \( \beta 5 \) and \( \beta 6 \) occurred weakly in a focal pattern, but only in the more primitive glomerular stages. The \( \alpha 8 \) subunit was present from the vascular stage onward and was expressed by the mesangial cells in adult glomeruli (Fig 8D). The integrin \( \alpha v \) subunit was detected earlier in development from the comma stage onward and later on in development it was expressed by both the GVEC and the mesangial cells (Fig 8E). No \( \alpha 4, \alpha 7B \) and \( \beta 4 \) were detected, but early in development \( \alpha 7B \) was weakly present in foci in the vessel walls in the kidney of the 1-month old newborn. Its distribution and expression increased during further development and aging (Fig 8C).

**DISCUSSION**

The evolution, composition and function of the GBM in the kidney has still not been fully unraveled. In the present concept, the GBM, at the interface between adjacent cells from epithelium and endothelium, is assembled from components derived from cells at both sides, but with the podocytes as the main contributors (Abrahamson and St.John, 1994). Podocytes, like the rest of the nephron, originate from the mesoderm. After the induction and polarization of the nephroblastema, transformation into tubular, parietal and visceral (podocytes) epithelium occurs. The mesangial and endothelial cells probably share a common progenitor from ingrowing vessels (Ekblom, 1981). Laminin, nidogen and collagen IV are essential for the integral development of a basement membrane. Nidogen has been attributed a role as an essential connecting bridge between laminin and collagen IV, thereby forming the basement membranes supramolecular structure (Ekblom et al., 1994; Aumailley et al., 1993; Fox et al., 1991). Nidogen is connected to laminin-1 by the \( \gamma 1 \) chain. The significance of laminin in the filtration process in the GBM has been shown in disease models in which antibodies against laminin induce proteinuria (Aten et al., 1995).
Since the contribution of the different cell types in the glomerulus to the formation of the GBM are difficult to study in vivo, we wished to perform in vitro studies. As no established human cell lines were available to us for this purpose, we used two rat cell lines: GVEC (derived from podocytes) and mesangial cells. Major differences between human and rat cell lines are not expected, but cannot be formally excluded. Because anti-mouse IgG crossreacts with rat IgG, rat kidneys could not be used for comparison with the in vivo expression and human kidney tissue was therefore used instead.

**ECM Components**

Mesangial cells produced laminin-1, nidogen and collagen IV, whereas GVEC produced laminin-5 and collagen IV but no laminin-1 or nidogen. The production of nidogen by mesangial cells, but not by GVEC, is in agreement with the results of in situ hybridization and Northern blot analysis in a mouse model, in which it was shown that nidogen is produced by mesenchymal cells but not by epithelia (Thomas and Dziadek, 1993). It thus seems that not all components necessary for GBM assembly are derived from the visceral epithelium, and that the contribution of the mesangial and perhaps endothelial cells (not tested in this study) to GBM formation is probably more important than was previously assumed.

Interestingly, both cell lines produced two unknown laminin isoforms. The α chain of one of them had a molecular mass of about 375 kDa (αx), which is most likely the equivalent of the α chain of the laminin isoform purified by Lindblom et al. (1994) from bovine kidneys. The α chain of the other isoform was approximately 395 kDa (αy). It is unlikely that this is the recently described human α4 chain since the molecular mass of this α chain is 200 kDa and it dissapears during development (Iivanainen et al., 1995). A chain of about 395 kDa has been found in cultured murine and bovine endothelial cells (Sorokin et al., 1994) which may be the same chain characterized in mice as the laminin α5 chain (Miner et al., 1995). It is also possible that the 375 kDa polypeptide represents a processed form of the 395 kDa chain. The production of two possibly new laminin isoforms by both GVEC and mesangial cells indicates that the GBM contains more laminin isoforms than just laminin-1 and laminin-3. The latter two are not present in the adult GBM as is shown by the observations mentioned later on.

In vivo laminin-5 was found in blood vessels, often in the hilus of the glomerulus and in the basement membrane of distal and collecting tubules, but not during glomerulogenesis. Therefore, the production of laminin-5 by GVEC may either be a phenomenon induced by cell culturing, or it may indicate that this laminin isoform is only transiently produced in nephrogenesis or only under pathophysiological circumstances.
In nephrogenesis, different developmental stadia are recognized by light microscopy, of which the comma-, the s-shaped body stage and the vascular-stage of the primitive glomerulus can be best distinguished. A temporarily controlled synthesis of different laminin isoforms is seen in nephrogenesis (Engvall et al., 1990; Virtanen et al., 1995), and supported by the results of our immunohistochemistry.

FIGURE 8 Distribution of integrin α3A (A), α6B (B), α7B (C), α8 (D) and αv (E) subunits in adult human kidney. The integrin α3A subunit is strongly expressed in glomeruli (G), especially by the podocyte (P) and more weakly by tubular epithelium (T). α6B is mainly expressed by tubular epithelial cells. In the glomerulus only a weak and focal staining is seen. α7B is present in extraglomerular vessels (EV), α8 in the mesangium (M) and αv in both the glomerulus, probably in all cell types (P = podocyte, M = mesangial cell) and the tubular epithelium (T). (See color plate V).
studies with antibodies against laminin chains in fetal, newborn and adult kidney.

As previously shown, we saw the laminin α1 chain appear when cell polarization takes place (Ekblom et al., 1990; Klein et al., 1990), but in contrast to Virtanen et al. (1995), we also found it to disappear from the comma stage onward. This discrepancy may be due to the different antibodies used. The laminin α1 chain is probably replaced by another α chain, e.g. by one of the newly described α chains (αx and/or αy) mentioned above. Furthermore, we showed the appearance of the laminin β2 chain from the vascular stage onward, probably replacing the β1 chain. The absence of the laminin β1 chain in adult glomeruli has also been shown by others (Miner and Sanes 1994; Noakes, 1995). There is evidence that the laminin β2 chain is produced by endothelial cells (Miner and Sanes, 1994). The laminin γ1 chain (Fig 6A-C) was present throughout development.

From the above we suggest that in the adult GBM αxβ2γ1 and αyβ2γ1 are important laminin isoforms and that they probably also play a role in glomerulogenesis.

**Integrins and Integrin Subunits**

In general, the expression pattern of the integrins in the glomerulus of the adult kidney were consistent with that found in cultured GVEC and mesangial cells.

Mesangial cells have been found to express the integrin α subunits, α1, α5 and α6. All these α subunits combine with β1 (Petermann et al., 1993). In addition, we found that the integrins expressed by mesangial cells include αvβ5 and αvβ6 in vivo, and αvβ3 and αvβ5 in vitro. The precipitation of α1β1 and the in vivo presence of α8, both phenotypical markers of smooth muscle cells, reflects the smooth muscle origin of the mesangial cells and thus its contractile abilities (Schnapp et al., 1995; Belkin et al., 1990; Glukhova et al., 1993).

Besides the expression of α3β1 reported for GVEC (Cybulsky et al., 1992; Adler, 1992), we found weak expression of α2β1 (Mendrick et al., 1995), and of α6Aβ4 and α6Bβ4, which are high affinity receptors for the produced laminin-5 (Niessen et al., 1994), and of αv, associated with an as yet unknown β subunit, possibly β8. Evidence for in vivo expression of these integrins on podocytes could not be obtained.

The integrins expressed by GVEC and mesangial cells are consistent with the ECM components they produce, which are ligands for these integrins. In most cells α1β1 and α2β1 both act as collagen receptors. α5β1 is a fibronectin receptor, αvβ5 and αvβ8 are vitronectin receptors and α8β1 and αvβ3 are fibronectin and vitronectin receptors (Hynes, 1992; Sonnenberg, 1993; Schnapp et al., 1995; Bossy et al., 1991). The integrins α3β1 and α6β1 are both laminin receptors. α3β1 recognizes laminin-5 and the laminin isoform present in the bovine kidney preparation (Delwel et al., 1994). Since no laminin-5 is present in glomeruli, together with the high level at which α3Aβ1 is expressed in the glomerulus, we suggest that α3Aβ1 is the main integrin receptor for the αxβ2γ1 and αyβ2γ1 laminin isoforms.

Cytoplasmic splice variant switches have been described in several organs during development (Cooper et al., 1991; Sutherland et al., 1993; Hierck et al., 1993). This indicates that this process may play an essential role in embryogenesis, although which role is still unclear. Therefore, we studied the cytoplasmic splice variants of α3 and α6 during glomerulogenesis. However, a switch was not detected. The only α3 splice variant found during glomerulogenesis and in adult glomeruli was α3A. The weak and focal expression of α6B in the glomerulus is probably a remnant of the tubulogenesis which precedes the actual glomerulogenesis.

**CONCLUSIONS**

In order to better understand which cells contribute to the composition of the GBM, we have investigated the production of ECM components and the expression of integrins by mesangial cells and GVEC grown in culture. Both cell lines were found to produce two new laminin isoforms (αxβ1γ1 and αyβ1γ1), of which one is probably already known as kidney laminin (αxβ1γ1). The laminin β2 chain is not
produced by GVEC or mesangial cells, although it is strongly expressed in vivo. The lack of production of nidogen by GVEC suggests that mesangial cells and possibly also endothelial cells, are essential sources for GBM production and assembly. Based on our findings and the literature, we postulate that the GBM in adult glomeruli is composed of laminin α3β2γ1, αγβ2γ1, nidogen, collagen IV, heparan sulfate proteoglycans and fibronectin. The integrins expressed on mesangial cells and GVEC is in accordance with the ECM components produced in vitro and compatible with the in vivo expression. α3Aβ1 appears to be the main integrin receptor in glomeruli for αβ2γ1 and αγβ2γ1.

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