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Expression of Extracellular Matrix Proteins in Basal Membranes During Fetal Nephron Development in Mice

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Abstract. In this study, we investigated the distribution of laminin, collagen type IV, nidogen and fibronectine during metanephric development in fetal mouse kidney by immunohistochemistry. Stain density of basement membranes of tubules, glomerules and mesangial matrix were compared in pre-capillary, immature glomerular and mature glomerular stages of fetal kidney. All the matrix proteins were strongly stained in precapillary stage. In immature glomerular stage, a strong staining was observed for fibronectin. Staining intensity was slightly decreased for the other proteins in this stage. In mature glomerular stage, diminished staining for all proteins was observed similar to the previous stage, except fibronectin. The strongest immunoreactions were found for fibronectin and nidogen in all investigated stages. In general, there was a similar staining intensity for all glycoproteins during maturation except for laminin. It was thought that the distribution of extracellular matrix molecules plays an important role for the kidney development. Interactions amoung these molecules probably crucial on cell behavior like migration, proliferation and differentiation in normal development of the nephron.

Keywords: Fibronectine, Nidogen, Laminin, Collagen type IV, Immunohistochemistry, Fetal Kidney, Mouse

Fare Embriyosu Nefron Gelişiminde Bazal Membrane Ekstrasellüler Matriks Protein Dağılımı

Özet. Çalışmamızda, fötal fare böbreği metanefrik gelişimde laminin, kollagen tip IV, nidogen ve fibronektin dağılımını imunohistokimyasal olarak inceledik. Tübül, glomerul ve mesangial matriks bazal membran boyanma yoğunluğu, fötal böbreğin pre-kapiller, olgunlaşmamış glomerül ve olgunlaşmış glomerül safhalarında karşılaştırıldı. Matriks proteinlerinin hepsi pre-kapiller safhada koyu boyandı. Olgunlaşmamış glomerül safhasında, fibronektin kuvvetli boyanırken diğer proteinlerin boyanma yoğunluğu azaldı. Olgunlaşmış glomerül safhasında ise fibronektin hariç diğer proteinlerin boyanma yoğunluğu olgunlaşmamış glomerül safhası ile benzerdi. İncelenen tüm safhalarda en yoğun immunreaksiyon nidogen ve fibronektinde bulundu. Genellikle, laminin hariç olgunlaşma boyunca tüm glikoproteinlerin boyanma yoğunluğu benzerdi. Bu, hücreler arası madde proteinlerinin böbrek gelişiminde önemli bir rol oynadığını düşündürmektedir. Muhtemelen bu moleküller arasındaki etkileşimler, normal bir nefron gelişiminde göç, çoğalma ve farklılaşma gibi hücre davranışlarında oldukça önemlidir.

Anahtar Kelimeler: Fibronektin, Nidogen, Laminin, Kollajen tip IV, İmmunohistokimya, Fötal böbrek, Fare

1. INTRODUCTION

Mammalian nephrogenesis starts in reciprocal interaction between undifferentiated mesenchyme and ureteric bud. Interactions among the extracellular matrix molecules provide the main factors for epithelial-mesenchymal differentiation in nephrogenesis. Spatiotemporal distribution of these molecules organizes development of metanephros.

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Development of mature kidney is comprised of three different spatiotemporal and regional developmental stages called pronephrosis, mesonephrosis and metanephrosis. Development of metanephrosis starts in 5th week for humans, 12th day for rat and 11th day for mouse and then it differentiates into mature and functional kidney [1-3]. This differentiation does not produce a mature kidney but provides two fetal tissues, metanephrogenic mesenchyme (MM) and ureteric bud (UB). Invation of MM into UB causes branching of UB and produces urinary collecting duct system [4-11]. Condensation of mesenchymal cells in this area helps differentiation of epithelia [3, 9, 12, 13] where they produce very specialized structures such as secreting nephron, glomerulus, proximal-distal tubules and the loop of Henle [1, 14]. The development of the nephron during this conversion passes through stages as differentiation of comma-shape from condensed mesenchyme, S-shape, precapillary, immature glomerulus and end up as mature glomerulus [15]. Renal glomerulus consists of two different cell layers, vascular endothelium and podocyts of visceral epithelium. Each of the epithelium formed from different origins where they supported by basal membranes.

Nephron differentiation during kidney development is a good example of epithelial-mesenchymal interactions. Matrix proteins that regulate maturation of basement membrane affect this differentiation [12, 16, 17]. Major glycoproteins (GPs) of basement membrane are the main factors for the organization of these interactions and regulate tissue growth and development [14, 18, 19]. Collagens, tenascin, nidogen, fibronectin are examples of mesenchymal extracellular matrix (ECM) proteins and type IV collagen, laminin and proteoglycan (PG) are integral basement membrane proteins which regulate metanephric development. Interactions among laminin, collagen IV, nidogen and other molecules play an important role for regulation of cell behavior through basement membrane [18, 20]. These interactions are affected by many molecules like cell adhesion molecules (CAM) and integrin receptors. Interactions between laminin and nidogen produce high affinity complexes in basement membranes by specific binding between the laminin gamma 1 chain and the G3 globule of nidogen.

Additional interactions among nidogen and type IV collagen, perlecan and other basement membrane components result in the formation of tertiary complexes between of these matrix components. Nidogen is highly susceptible to proteolytic cleavage, and binding to laminin protects the nidogen from degradation. Nidogen is considered to have a crucial role as a link protein in the assembly of basement membranes [21, 20]. Fibronectin is also an important protein for cell-cell, cell-matrix interactions, mesenchymal cell condensation. It decreases as cyto-differentiation proceeds [22]. Therefore, ECMs affect cell behavior by different type interactions and specific functions [23].

The aim of this study is to investigate the distributions of laminin, collagen type IV, nidogen and fibronectin during fetal kidney development. We compared their changes in pre-capillary, immature glomerular and mature glomerular stages.

2. MATERIAL AND METHODS

30 female and 15 male BALB/c type mice were used in this study. Following a one-week isolation period in separate cages, all animals were put in a single cage for a night to get the females pregnant. The age of the embryos was determined from the vaginal plug, the appearance of which was designated as embryonic day 0. The females were separated again the next day and were followed-up for pregnancy. Samples were investigated at embryonic days (E) E16, E18 and newborn. Pregnant mothers and postnatal pups were anaesthetized with directly ether (Merck, Germany).

Fetal and newborn kidneys were immersion fixed in Saint-Marie's solution for 24 h at 4°C and processed [24-26] for paraffin procedure. All samples of 5 μm-thick serial sections were taken on poly-l-lysine coated slides (Sigma, U.K.). The sections were deparaffinized and washed with a decreasing series of ethanol, after then washed in distillated water. The section were delineated with a DAKO pen (Sigma, U.K.) and incubated with %0.1 tripsin-%0.1 Ca Chlorid. After primary (Laminin-1/100 Sigma L20202; collagen type IV-1/10 Sigma C0543; nidoge-1/6 Chemicon-mab 1946; Fibronectin-1/100 Neomarkers, Fremont, CA, USA) and secondary antibody application, sample were visualized by peroxidase method and peroxidase (HRP) activity was detected using H_2O_2 (0.03%) and 3-amino-9-ethylcarbazole (ACE) staining. The sections were counterstained with Mayer's hematoxylin and were covered with mounting medium. Negative control sections were included where the primary and/or secondary antibody was omitted and replaced by normal serum alone. All samples were investigated under light microscope (Olympus BX50, Tokyo-Japan) and photographs (Fujifilm-ASA 100) were taken.

3. RESULTS

In this study, fetal kidneys at 16^{th} and 18^{th} days and new borne kidney were studied in sagittal sections. Three different, such as pre-capillary (Fig. 1a), immature glomerular (Fig. 1b) and mature glomerular (Fig. 1c) stages were distinguished. Localization of extracellular matrix components such as laminin, type IV collagen, nidogen and fibronectin were determined by immunohistochemistry using specific primary antibody in three different stages. Since nephrogenesis is a continuous phenomenon in the metanephric fetal kidney, nephron development could be distinguished at different stages where immature types observe at periphery.

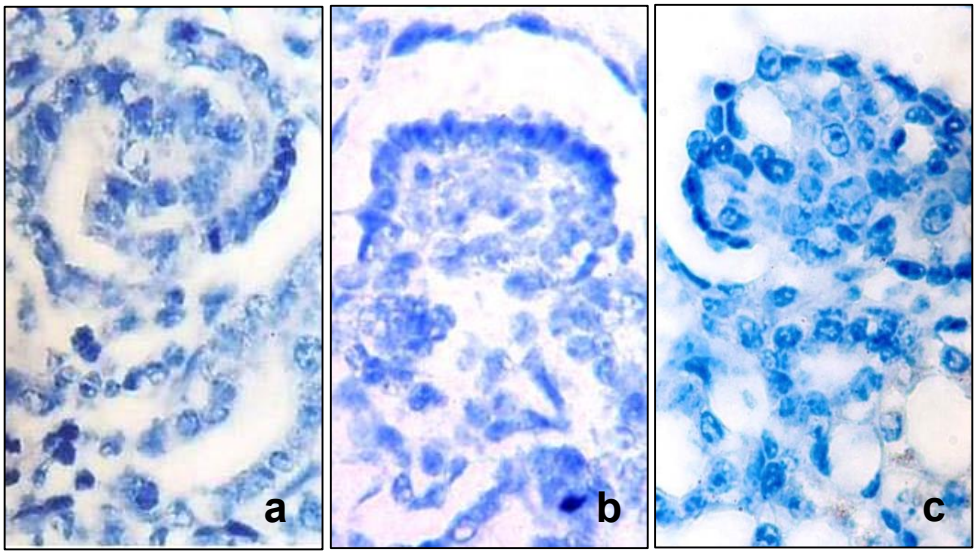

Figure 1. Negative control obtained by incubating renal tissue sections with a non-immune mouse serum. a: precapillary stage, b: immature glomerular stage, c: mature glomerular stage (a, b, c: x100).

The highest immunoreactivity among the studied extracellular matrix protein was found for fibronectin. All the basement membranes were strongly stained in compared three stages. The basal membranes surrounding the ingrowing mesenchymal components destinated to become renal tubule endothelia and mesangial cells, in pre-capillary stage (Fig. 2a) nearly identical positive staining for fibronectin (Fig. 2a, b). Increased staining density was found at the same area in immature and mature glomerular stages (Fig. 2b, c).

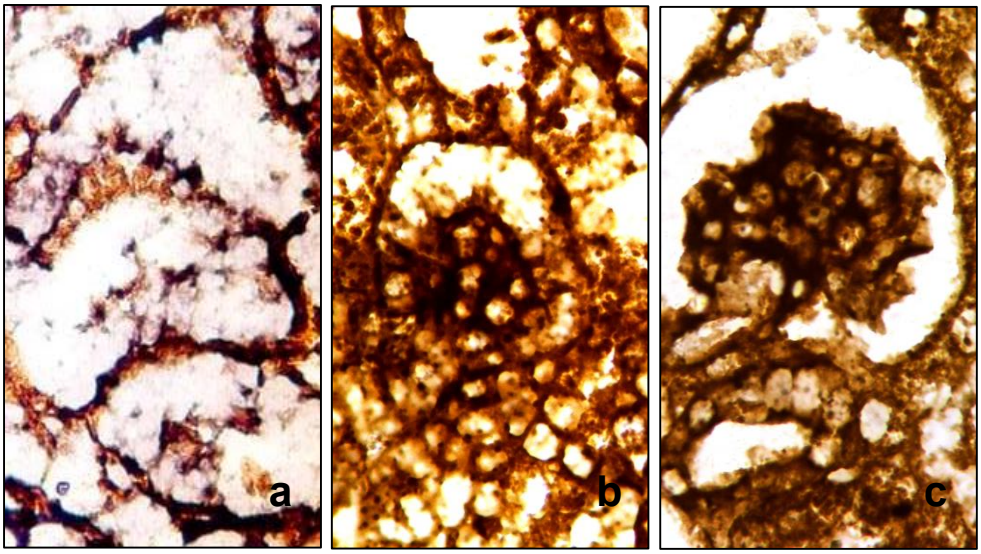

Figure 2. Fibronectin immunoreactivity in whole basement membrane, mesangial matrix of precapillary stage (a), immature glomerular stage (b) and mature glomerular stage (c) are strongly stained positive (a, b, c x100).

A second strong immunoreactivity was found for nidogen. Bowman's capsule, tubules, mesangial areas and glomerulus, were showed a strong immunoreactivity for nidogen (Fig. 3a, b) like to fibronectin in precapillary and immature stages. In mature stage reduced immunoreactivity was observed (Fig. 3c). This is contrast for the fibronectin staining.

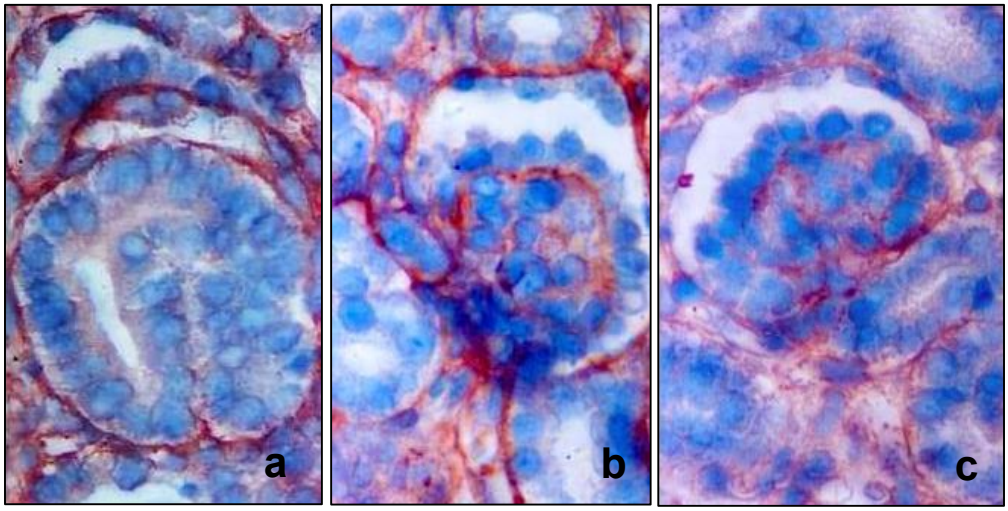

Figure 3. Nidogen immunoreactivity in GBMs of precapillary stage (a), immature glomerular stage (b) are strongly stained positive. This pattern of staining persists in the mature glomerular stage (c) (a, b, c x100).

The staining of laminin immunoreactivity was found very similar for nidogen in pre-capillary and immature stages (Fig. 4a, b). Decreased staining density was found at the same area in immature glomerular stage (Fig. 4b). Furthermore, a number of dot-like staining particles were seen among the cells which will be formed as glomerulus. In mature glomerular stage, the immunoreactivity was slightly increased in density but being still less amount than those found in first two stage (Fig. 4c). A strong positive staining was observed especially at the lumen side of proximal tubule.

Staining intensity of the basal membranes for collagen type IV was similar to that of found for all the other proteins. In the immature glomerular stage (Fig. 5b), collagen type IV immunoreactivity was decreased in general. Visceral layer of the Bowman's capsule and mesangial region showed diminished staining feature (Fig. 5b). But a strong positive reaction spots at the surface of the parietal layer of the Bowman's capsule was obtained. In the mature glomerular stage, reduced staining density was observed in the basement membranes. There was also some staining in extra cellular matrix where localized out of the basement membrane (Fig. 5c).

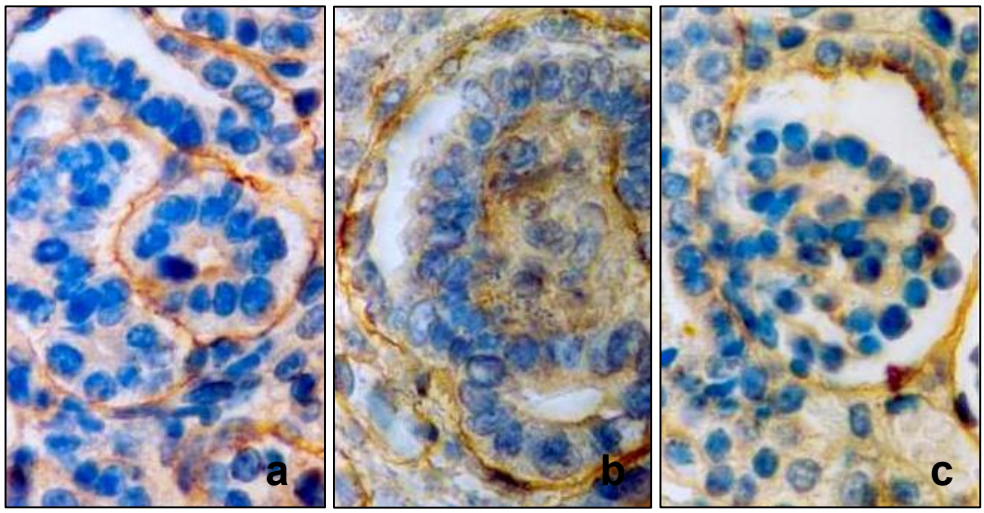

Figure 4. Laminin immunoreactivity showed positive staining around the epithelial component in pre-capillary stage (a), immature glomerular (b) and mature glomerular (c) stage. The growing mesenchymal component and mesangial region in mature glomerulus were also positive (a, b, c x100).

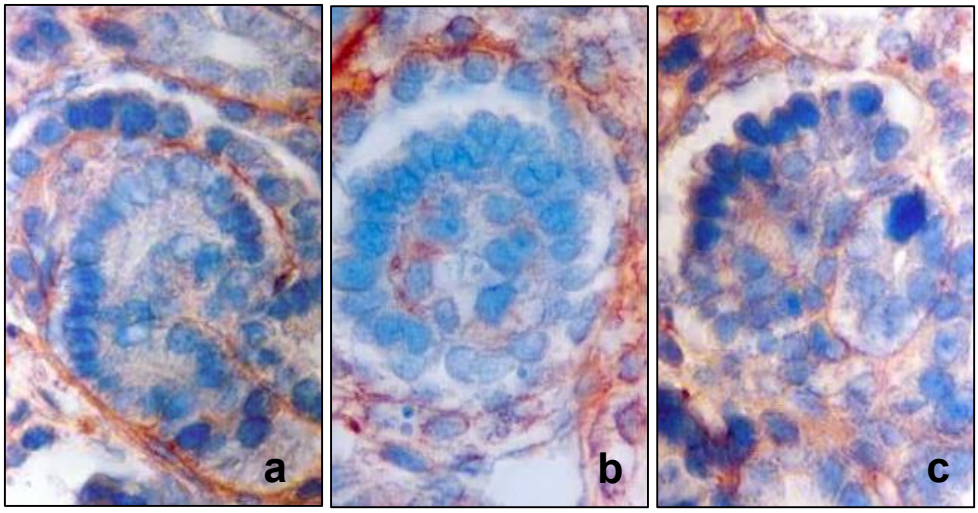

Figure 5. Type IV collagen immunoreactivity showed positive staining around the epithelial component in pre-capillary stage (a), basement membrane of the parietal layer of Bowman's capsule (a, b, c x100).

As a result, fibronectin immunoreactivity was strongly stained in whole basal membranes and mesangial matrix in compared three stages. Second highest immunoreactivity was found for nidogen particularly in first two stages. Laminin immunoreactivity was observed nearly similar to that found for nidogen expect in mature stage. In general, the weakest staining was found for collagen type IV in all areas of the developmental stages compared to the other proteins.

Immunoreactivity staining ranking from the highest to the low level can be as following: fibronectin > nidogen > laminin > collagen IV (Table 1).

Table 1. Immunoreactivity of ECM proteins in three different developmental stages.

	Pre-capillary	Immature glomerul	mature glomerul
Fibronectin	+++++	+++++	+++++
Nidogen	+++++	++++	+++
Laminin	+++++	++++	+++
Collagen type IV	+++++	++	+++

Although a strong immunoreactivity was found in precapillary and immature stages for all proteins the highest and lowest reactions were observed for fibronectin and collagen IV, respectively. The order of proteins like fibronectin > nidogen > laminin > collagen IV. In mature stage was still found for fibronectin, the lowest reaction was found for laminin.

4. DISCUSSION

Metanephric differentiation occurs a highly controlled series of morphogenetic and differentiation events that starts in reciprocal inductive interactions. Primitive mesenchyme in fetal kidney is induced by branching UB and the conversion of mesenchymal cells into epithelial structure occurs. The epithelial cells of the ureteric branches induce the surrounding mesenchymal cells to condense like epitelia and give rise to most part of the nephron. Developmental stages along the metanephrogenic pathway is initiated and organized by the expression of new combination of ECM elements and factors secreted by the mesenchyme.

It has been found in some of the previous studies that, most of the ECM proteins were expressed after induction [16]. Fibronectin alongside laminin plays a role for the regulation of cell migration [27, 28] where these molecules have to be hydrated by glycosaminoglycans. Collagen IV has receptors on the cell surface for heparin and integrins, which affect cell cytoskeleton and also binds laminin for the maintenance of the basement membrane [29, 30]. Temporal changes of these protein expressions can originate from structural differences as it was shown for collagen IV [31, 32] and heparin sulphate proteoglycan [33]. Therefore, it is possible to explain the temporal and differential changes in these protein expressions by structural alterations [34, 35]. Previous studies show that, staining may be altered depending on many factors, technical reasons for these changes are still debated because of alter in staining of collagen IV and fibronectin using monoclonal antibodies [25, 36]. These studies show that different types of ECM molecules interact with different type of molecules and their functions alter by this way.

It has been shown that, laminin, type IV collagen [28] and fibronectin [37, 27, 38] affect the migration of the cells and can be found around the mesenchymal cells in kidney [39]. Moreover, the basement membranes in all stages of nephron development have these molecules, which sometime their distribution is irregular and heterogenic. Laminin, collagen type IV and nidogen distribution were found regular, continue in the basement membrane of mature nephron whereas fibronectin was situated on the lateral side of cell-cell contact and around the glomerular mesenchymal cells [2, 25, 27, 39-41]. Our immunohistochemistry results for laminin, nidogen and fibronectin were similar to the previous studies in this respect. However, collagen type IV drastically decreases in mesangial matrix and in both glomerular and tubular basement membranes in mature glomerular stage. This present result was also supported by Harvey et al., (1998)[42] who showed that Collagen IV was expressed at the early stage of dog glomerular development but disappeared at the later stages due to structural changes instead of technical reasons.

In undifferentiated mesenchyme, collagen IV, laminin and heparan sulphate proteoglycan were absent. In agreement with our study, in differentiated mesenchyme, type IV collagen was reduced during maturation. Similarly, fibronectin distribution was found heterogeneous and it was strongly stained in immature glomerular and mesenchymal basal membrane. But this staining was also reduced in glomerular basal membrane with maturation. It has been shown that some mesenchymal proteins, for instance, interstitial collagens and splash variations of fibronectin were reduced after maturation of the nephron and the proteins like tenascin temporarily appeared at condensed or S-shaped stage. Nidogen and its interaction with laminin play a crucial role during basement membrane formation of nephrogenesis. Laminin expression also depends on age, species and stage. In addition, depending on transient expression of laminin epitops immunoreactivities changes in glomerular basal membrane [43]. These molecules also interact with growth factors which affect metanephric kidney formation. Moreover, cell adhesion molecules as integral membrane proteins, affect metanephric development by interacting either with other adhesion molecules or with cell cytoskeleton [44].

ECM proteins are important for the development and maintenance of basement membrane, which is crucial for the development of the nephron. Since, the cells give information to each other by ECM molecules [14, 45-48] these proteins are also important for the integrity of the kidney tissues and behavior of the cells in these tissues.

Determination of their roles in development and epithelial-mesenchymal interactions are difficult to investigate because of their complex structures, their interaction with other molecules and growth factors and their effects on signal transduction. Our results show that their expressions

are heterogeneous and sometimes temporary. These results give clue for their functions and bring many questions for further studies. These studies will bring many benefits for the treatment of developmental kidney problems and disease in adults.

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