Three-dimensional Analysis of Nephrogenesis in the Neonatal Rat Kidney: Light and Scanning Electron Microscopic Studies

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Summary. In order to clarify the process of renal development more precisely than previously, the present study observed the rat neonatal kidney by scanning electron microscopy (SEM) of KOH digested tissue as well as by light microscopy of plastic sections. In the subcapsular region, aggregation of the mesenchymal cells was closely associated with the upper side of the ureteric duct ampulla. These mesenchymal cells projected a number of fine irregular processes at the basal portion facing the ureteric duct. A spherical cluster transformed from the mesenchymal cell aggregation was found on the lower side of the terminal ampulla, and was differentiated into the renal vesicle. Some cells at the top of the renal vesicle formed a cone-shaped projection and invaded the ureteric duct ampulla, forming a connection with it. In the advanced stage, a shallow transverse cleft appeared on the outer lateral side of the renal vesicle, and a second cleft was formed on the opposite side close to the junction between the renal vesicle and the ampulla. As the two clefts deepened, the vesicle assumed the well-known S-shaped body. In the advanced S-shaped body, the lower limb became cup-shaped, while the segment between the middle and lower limbs of the “S” elongated to form a tubular structure (i.e., the prospective proximal tubule and Henle’s loop). The upper limb of the “S” also increased its length to form a distal tubule. The middle limb of the “S”, however, was attached firmly to the cup-shaped lower limb (i.e., the prospective renal corpuscle) and was considered to become the macula densa of the mature nephron. In the maturing renal corpuscle, irregularly shaped cells were observed as a sheet-like aggregation at its vascular pole and were continuous with the vascular smooth muscle cells. These findings will help toward a better understanding of the morphological complexities of nephrogenesis.

Understanding the structure of the developing nephron is important for studies on the organogenesis of the metanephric kidney. In light microscopic studies, OSATHANONDH and POTTER (1963, 1966a, b) first clearly showed the basic process of nephrogenesis by means of a fractionated microdissection of individual nephrons after acid maceration of the whole human embryonic kidney. On the other hand, transmission electron microscopic studies by JOKELAINEN (1967) and KAZIMIERCZAK (1971) provided information on the ultrastructure of the developing nephron. These early findings were accordingly considered by SAXEN (1987), who studied developmental changes in the metanephric mesenchyme by using time lapse microscopy of the cultured kidney and published a monograph on the organogenesis of the organ.

It is therefore apparent that the nephron in the metanephric kidney is produced as an aggregation of the metanephric mesenchymal cells adjacent to the ureteric duct. They subsequently acquire “epithelial” properties and form a spherical cluster, or the renal vesicle. After this mesenchyme-epithelium conversion, the renal vesicle gradually changes its shape from spherical to S-shaped and is finally differentiated into the mature nephron consisting of the renal corpuscle and a tubule.

Despite of these studies, several questions remain unresolved due to previous methodological limitations to the three-dimensional analysis of the structures concerned. To overcome this problem, we recently applied scanning electron microscopy (SEM) of KOH-digested tissues to studies of the neonatal mouse kidney and demonstrated that this method is suitable for investigating the shape of developing nephrons directly and three-dimensionally (IINO et al., 2001). Thus, the present study observed the rat neonatal

Fig. 1. Light micrograph of the neonatal rat kidney stained with toluidine blue. Developing nephrons at various stages are seen in the renal cortex. *Arrows* indicate a cluster of mesenchymal cells around the ureteric duct (*U*). *GC* globular condensation of mesenchymal cells, *RV* renal vesicle, *S* S-shaped body, *G* glomerulus at the capillary loop stage. $\times 480$

kidney by SEM of KOH-digested tissues as well as by light microscopy of plastic sections. The purpose of the present study is to clarify the process of the renal development more clearly and three-dimensionally than previous research.

MATERIALS AND METHODS

Newborn Wistar rats of both sexes were anesthetized by ice and perfused through the heart with 2% glutaraldehyde in 0.1 M phosphate buffer at pH 7.4. Immediately after perfusion, the kidneys were removed from the body and immersed in the same fixative for

more than 24 h at 4°C. For light microscopy, the kidney was postfixed with 1% osmium tetroxide in the same buffer, dehydrated in a series of ethanol, and embedded in epoxy resin according to conventional procedures. Tissue sections, about 1 μ m thick, were obtained with an ultramicrotome, stained with toluidine blue, and observed with a light microscope. For SEM, the glutaraldehyde-fixed kidney was entirely placed in a 30% KOH aqueous solution for 8–10 min at 60°C in order to remove extracellular matrices such as collagen fibrils and basal laminae (USHIKI and MURAKUMO, 1991; IINO et al., 2001). After KOH treatment, the kidney was washed several times in the buffer and divided into small pieces with a fine needle and forceps under a dissecting microscope. These tissues were conductive-stained according to MURAKAMI (1973); they were treated with 1% tannic acid aqueous solution for 1 h, rinsed in distilled water for 1 h, and immersed in 1% osmium tetroxide for 1 h. The tissues were rinsed in distilled water several times, dehydrated through a graded series of t-butyl alcohol and directly frozen on a cooling metal plate followed by evaporation to 0.1 Pa in a vacuum chamber (INOUE and OSATAKE, 1988). The freeze-dried specimens were mounted on aluminum stubs with adhesive tape, coated with platinum-palladium in an ion coater, and observed in a Hitachi S-2700 scanning electron microscope at an accelerating voltage of 10 kV.

RESULTS

Light microscopy of plastic sections revealed that the cortex of the newborn rat kidney contained developing nephrons at various stages (Fig. 1). The nephrons at earlier stages were located at the subcapsular region, while those at later stages were found in the deeper portion of the cortex. In KOH treated specimens, the shape of developing nephrons was observed directly by SEM because extracellular matrices surrounding the nephrons were effectively removed by this treatment.

The ureteric duct was observed as a straight tubule, about 15–30 μ m in diameter, and ended as a swollen terminal or ampulla in the subcapsular region of the renal cortex (Fig. 2a, b). Each ampulla usually received two or three connecting pieces of developing nephrons to form the arcade-like structure shown by previous authors (OSATHANONDH and POTTER, 1963; NEISS, 1982) (micrograph not demonstrated). A distinct aggregation of mesenchymal cells was usually found in close association with the ampulla of the ureteric duct (Fig. 2a, b). In specimens adequately

Fig. 2. Early condensation of mesenchymal cells (*EC*) around the ampulla of the ureteric duct (*U*). **a.** Light micrograph showing a section profile of mesenchymal cells (*EC*) which aggregate around the terminal ampulla of the ureteric duct (*U*). **b.** Scanning electron micrograph of a similar portion of Figure 2a. Irregular-shaped mesenchymal cells (*EC*) are arranged around the ureteric duct (*U*). **c.** In this specimen the ureteric duct has accidentally been removed, and the basal portion of the condensed mesenchymal cells (*EC*) is clearly demonstrated. **d.** Closer view of a part of Figure 2c. Aggregated mesenchymal cells have numerous fine irregular processes at the basal portion facing to the ureteric duct. a: $\times 1,300$, b: $\times 1,200$, c: $\times 1,100$, d: $\times 3,100$

cracked at the junction between the mesenchymal cells and the ureteric duct, each of the former cells projected numerous fine irregular processes in the basal portion facing the ureteric duct (Fig. 2c, d).

The mesenchymal cell aggregation at the advanced stage was observed as a spherical cluster facing the underpart of the terminal ampulla, and appeared to differentiate into the renal vesicle (Fig. 3). Under the light microscope, the renal vesicle was found to be composed of radially oriented, columnar cells (Fig. 3a). By SEM, each cell in the vesicle was polygonal but rather irregular in shape and had a few micropro-

jections on its surface (Fig. 3b). In the developed renal vesicles, some cells in the upper part of the vesicle formed a cone-shaped protrusion, which extended toward the ureteric duct ampulla to make an insertion into it. Light microscopy of toluidine blue sections showed that a clear distinction existed between the cells derived from the renal vesicle and those derived from the ureteric duct due to the difference in staining intensity between them. The renal vesicle was thus tightly connected with the ureteric duct, and now revealed a distinct lumen within the vesicle (Fig. 3c). By SEM, a deep ridge separating the ampulla and

Fig. 3. Globular mesenchymal condensation (GC) and renal vesicles (RV). **a.** Globular condensation (GC) is found just beneath the terminal ampulla. Note also the early condensation of the mesenchymal cells (EC) around the ureteric duct. V blood vessel. **b.** Scanning electron micrograph corresponding to Figure 3a. GC globular condensation, U ureteric duct. **c.** The renal vesicle (RV) has a cone-shaped projection (*) which penetrates into the ampulla of the ureteric duct (U). EC early mesenchymal cell condensation, V blood vessel. **d.** Scanning electron micrograph corresponding to Figure 3c. The renal vesicle (RV) is connected with the ureteric duct (U). Blood vessels are found around the renal vesicle. Note the transverse ridge (arrow) separating the ampulla and renal vesicle. Arrowhead indicates the prospective transverse cleft of the S-shaped body. a: $\times 1,150$, b: $\times 1,300$, c, d: $\times 1,100$

Fig. 4. S-shaped bodies (S) at various stages of development. **a** and **b.** Immature S-shaped bodies (S). A shallow cleft (*arrowhead*) is found at the lower lateral portion of the S-shaped body. Note a blood vessel (V) which is in close vicinity of this cleft. EC early mesenchymal cell condensation, U ureteric duct. *: connection between the S-shaped body and ureteric duct. **c** and **d.** S-shaped bodies at the advanced stage. Cellular components at the lower portion of this body differentiate into an inner (IL) and an outer (OL) layer, suggesting its transformation to the renal corpuscle. Blood vessels (V) are observed at the edge of the lower cleft of the S-shaped body. EC early mesenchymal cell condensation, U ureteric duct. *: connection between the S-shaped body and ureteric duct. *Arrowheads* and *arrows* indicate the lower and upper cleft of the S-shaped body, respectively. a: $\times 800$, b: $\times 1,200$, c: $\times 1,000$, d: $\times 1,600$

Fig. 5. S-shaped bodies and immature glomerular epithelial cells. **a.** In a fractured specimen, the lower limb of the S-shaped body consists of inner thick (*IL*) and outer thin (*OL*) layers, indicating that they are in the process of differentiation into glomerular epithelial cells and Bowman's capsules, respectively. *M* middle limb of the "S", *UL* upper limb of the "S". *U* ureteric duct. **b.** The advanced S-shaped body. Note the segment (☆) between the middle and lower limbs of the "S" which expand to form a tubular structure. *B* Bowman's capsule, *M* middle limb of the "S", *D* prospective distal tubules, *U* ureteric duct. **c-e.** (Continued on the next page ^)

Fig. 6. S-shaped bodies at advanced stages. **a.** The inner layer of the lower limb has differentiated into glomerular epithelial cells (*GE*), which resemble a cluster of grapes. Note blood vessels (*V*) in the lower cleft. **b.** The segment between the middle (*M*) and lower limbs (*B*) elongates to form a prospective proximal tubule (*P*). Whereas, the upper limb of the S increases its length to become a distal tubule (*D*). *a:* $\times 1,400$, *b:* $\times 1,000$

renal vesicle was observed at the connection site. Small vessels consisting only of endothelial cells were often located around the renal vesicle (Fig. 3d).

At the advanced vesicular stage, some cells on the outer lateral surface of the renal vesicle became flattened and formed a shallow transverse cleft (Fig. 4a, b). On the other hand, a second cleft appeared in the upper portion of the opposite side (i.e., the inner lateral surface) close to the junction between the renal vesicle and the ampulla (Fig. 4c, d). As the two clefts deepened, the vesicle formed the well-known S-shaped body (Fig. 5a, b). Endothelial tubes of small vessels were often located near the deep outer crevice

at the lower portion of the renal vesicle (Figs. 4d, 5a, b). At this stage, the lower limb of the "S" became flattened like a hemisphere cup as if surrounding the middle limb of the "S". A thin sheet consisting of vessels and their associated cells was usually sandwiched between the middle and lower limbs (Fig. 5e). In adequately cracked specimens, the lower limb at this stage already consisted of an inner thick, and an outer thin layer; the inner thick layer was composed of prospective glomerular epithelial cells arranged like a cluster of grapes, while the outer thin layer comprised flat shaped cells of Bowman's capsule (Fig. 5a, c).

Fig. 5 c. The lower limb of the S-shaped body, where the outer thin layer (i.e., Bowman's capsule, *OL*) has been accidentally partially peeled off. The inner layer of the lower limb (*IL*) consists of both flat and cuboidal epithelial cells. **d.** Closer view of a part of Figure 5c. Note cellular margins (*arrowheads*) of epithelial cells. **e.** A cluster of blood vessels with irregular-shaped cells (*arrows*) is shown in the lower cleft of the S-shaped body. *B* Bowman's capsule, *D* prospective distal tubules. *a, b:* $\times 900$, *c:* $\times 1,350$, *d:* $\times 4,700$, *e:* $\times 1,150$

Fig. 7. Renal corpuscle in the capillary loop stage. **a.** Light micrograph of the renal corpuscle in the capillary loop stage. Glomerular epithelial cells (*GE*) are round to square in shape. Extraglomerular mesangial cells (*arrowheads*) are seen in the juxtaglomerular zone. **b.** Scanning electron micrograph of the renal corpuscle in the capillary loop stage. *GE* glomerular epithelial cells. **c** and **d.** Afferent (*A*) and efferent (*E*) arterioles are seen in the vascular pole of the developing renal corpuscles. In these renal corpuscles, irregularly shaped cells (*) form a sheet-like aggregation at the vascular pole. These cells are continuous with the vascular smooth muscle cells. **a:** $\times 1,400$, **b:** $\times 1,600$, **c, d:** $\times 1,200$

In the advanced S-shaped body, the short segment between the middle and lower limbs of the “S” expanded at first and then elongated to form a tubular structure (Fig. 5b). This portion was considered to become a proximal renal tubule and Henle’s loop. On the other hand, the upper limb of the “S” increased its length to form a distal renal tubule (Fig. 5b). During the elongation of the proximal and distal renal

tubules, the middle limb of the “S” seemed to attach firmly to the lower limb (i.e., the prospective renal corpuscle); this portion was considered to become the macula densa of the mature nephron (Figs. 4d, 5a, b, 6b).

Afferent and efferent arterioles were observed at the vascular pole of the developing renal corpuscles, which had glomerular capillaries and spherical epi-

thelial cells enclosed by the Bowman capsule (Fig. 7a, b). In these corpuscles, irregularly shaped cells formed a sheet-like aggregation at the vascular pole (Fig. 7c, d). Transitional forms between these cells and vascular smooth muscle cells were present in the walls of both afferent and efferent arterioles. As the renal corpuscles developed, cellular aggregation increased in density at the vascular pole to form extraglomerular mesangial cells.

DISCUSSION

The present study is the first to succeed in demonstrating three-dimensionally by SEM the process of the renal development from mesenchymal condensation to the capillary loop stage. In the present study, we used the kidney of neonatal rats, one week after birth, because in these animals developing nephrons at various stages are simultaneously found in the kidney (VESNA and SPOMENKA, 1980). The KOH treatment used in the present study was originally introduced by MILLER et al. (1982) and has been applied by our research group to the three-dimensional observations of various tissues and organs hidden in the extracellular matrices (USHIKI and IDE, 1987, 1988; USHIKI and MURAKUMO, 1991; USHIKI and WATANABE, 1997; HIGUCHI et al., 2000). As we recently introduced, this method is also suitable for investigating the shape of developing nephrons directly and three-dimensionally (INO et al., 2001).

The current consensus on morphological features of the developing nephrons is based on findings by OSATHANONDH and POTTER (1963, 1966a, b), who first observed under the light microscope the shape of nephrons isolated from acid macerated human kidneys. Our SEM findings have clearly shown that their classical findings are basically correct. Using modern techniques, however, we have observed the three-dimensional structure of developing nephrons more clearly and precisely than before. Figure 8 is a schematic representation of our results on the shape of developing nephrons at various stages.

The present study has clarified the presence of numerous fine projections of mesenchymal cells facing the terminal ampulla of the ureteric duct in the portion of the early mesenchymal condensation. These projections have probably developed as the result of the active direct interaction of the mesenchymal cells with the ureteric duct epithelium. Recent studies have shown that cell adhesion during nephrogenesis is not merely a function of the extracellular matrix but is also dependent on cell adhesion molecules found on the cell surface (VESTWEBER et al., 1985; ROSENBERG

Fig. 8. A schematic drawing of rat nephrogenesis. I: early mesenchymal condensation, II: spherical cluster of the mesenchymal cells, III: renal vesicle, IV: immature S-shaped body, V: advanced S-shaped body, VI: a nephron at the capillary loop stage. An *arrow* indicates the connecting site between the renal vesicle and the ureteric duct. An *arrowhead* indicates a shallow transverse cleft at the outer lateral surface of the advanced renal vesicle. *V* blood vessel, *M* the middle limb of the "S" (prospective macula densa). A *star* indicates the short segment between the middle and lower limbs of the "S". *D* prospective distal tubule, *P* prospective proximal tubule and *B* Bowman's capsule.

et al., 1997; MAH et al., 2000). It seems reasonable to presume that certain adhesion molecules may be specifically expressed at the very sites of these projections, although immunohistochemical studies will be needed to determine this point.

The mesenchymal cell aggregation changed its position from the upper to the under side of the

ampulla, to subsequently form the renal vesicle. The mechanism of this movement is still unknown; one possibility might be that the mesenchymal cells may actively move downward along the surface of the ampulla, while the other would be that the mesenchymal cell aggregation may be positioned as a result of the active elongation of the ureteric duct ampulla.

The renal vesicle or, as previously designated, the "comma-shaped body" is usually considered to be positioned free from the ureteric duct ampulla (e.g., see Figure 120 by SAXEN, 1987). However, we have clearly demonstrated that the renal vesicle is already connected with the ureteric duct ampulla by a mesenchymal cell cone inserted into the ureteric duct epithelium. Although a tiny projection on the top of the renal vesicle was previously correctly illustrated by OSATHANONDH and POTTER (1963) (see Figure 2B in their paper), they made no mention of it in their text. It should be also noted that in their illustration the renal vesicle, including the projection, was free from the ureteric duct ampulla, probably because the vesicle was mechanically dislocated in the course of microdissection of the acid macerated tissues. The phenomenon of the mesenchymal cell mass inserting or migrating into the ampulla is highly important when considering the mode of connection between these two components. This finding suggests that the connecting piece of the maturing nephron is composed of a mixture of ureteric duct cells and metanephrogenic mesenchymal cells, as supposed by several previous investigators (OSATHANONDH and POTTER 1963; OLIVER 1968; KRIZ 1972; NEISS 1982).

The present study has demonstrated precisely and three-dimensionally the structural change of the S-shaped body during differentiation of the nephron. The most noteworthy finding is that the middle limb of the S-shaped body tightly attaches to the prospective renal corpuscles during development and appears to finally become the macula densa of the maturing nephron; this also indicates that the lower limb produces the renal corpuscle and renal tubules from the proximal convoluted tube to the macula densa, and the upper limb becomes the distal tube far from the macula densa. As far as we know, the contribution of the middle limb to the macula densa was only briefly suggested by POTTER (1972). Our finding provides an embryological answer to the enigma of why the renal tubule returns to the renal corpuscles of the same nephron forming the macula densa at the site. The mechanism of the tight attachment of the middle limb to the lower limb remains unknown. Since there is a sheet of mesenchymal elements as well as basal laminae of both the middle and lower limb epithelium, extracellular matrices such as laminin and

fibronectin may be related to the maintenance of the attachment.

Regulation of the microvessel assembly in the developing renal corpuscles has not been clearly defined until today: one author postulated that the vasculogenic processes of the renal microvessel assembly were involved (ROBERT et al., 1998), while others insisted there were angiogenic processes (SARIOLA et al., 1983). In this study, we have shown that endothelial tubes either with or without periendothelial cells were detected at the early vesicular stage around the pretubular mesenchymal cell mass. These vessels were sprouts of preexisting large vessels, as demonstrated by previous authors by confocal laser scanning microscopy of the neonatal rabbit kidney (KLOTH et al., 1994, 1997); they demonstrated that endothelial tubes stained with monoclonal antibodies (Enpo 1 and EC 1) were found around the ureteric duct ampulla and renal vesicle. The presence of vessels near the deep transverse crevice of the vesicle may indicate that the endothelial tubes have invaded into the cleft to form glomerular tufts.

Recently, a few SEM studies have reported on the shape of the mesangial cells at the vascular pole of the mature nephrons (TAKAHASHI-IWANAGA, 1991; 1992) and immature nephrons (IINO et al., 2001). As shown in our previous studies of the mouse neonatal kidney (IINO et al., 2001), the intermediate form between extraglomerular mesangial cells and contiguous vascular pericytes was found at the vascular pole of developing renal corpuscles. In their gene-expression studies of the mouse kidney, LINDAHL et al. (1998) stated that mesangial cells were derived from perivascular smooth muscle cells, and that platelet-derived growth factor (PDGF)-B/PDGF-receptor β signaling controlled the differentiation from smooth muscle cells into mesangial cells. Pathologically, for the repair of the mesangium following antibody-induced mesangiolysis in rats, new mesangial cells were considered to be recruited from the juxtaglomerular area in a process of proliferation and migration (HUGO et al., 1997). The contractility of mesangial cells can also be better accounted for if they may be regarded as specialized vascular smooth muscle cells (SCHLONDORFF, 1987).

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