Neuronal characteristics in embryonic renal stroma

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ABSTRACT The metanephric mesenchyme is considered a homogeneous population of predetermined, but pluripotent cells with a nephrogenic bias. By an inductive stimulus, the mesenchyme is programmed to differentiate into the various epithelial phenotypes of the secretory nephron. A fraction of the mesenchymal cells, however, remains in the interstitium between the nephrons and differentiates into spindle-shaped, clear-cyttoplasmic renal stroma. We have analyzed the molecular nature of these cells in order to discover the specific cell types that could be involved in the morphogenetic processes during kidney differentiation. In situ hybridization reveals neurofilament light protein mRNA, and immunohistology shows neurofilament light and medium proteins in the stromal cells around kidney tubules. By immunohistochemistry, these peritubular stromal cells can be distinguished from the neuronal cells of the renal microganglion: the peritubular stromal cells are neurofilament-positive but L1 neural cell adhesion protein-negative, whereas the neuronal cells with axonal extension are both neurofilament-positive and L1 neural cell adhesion protein-positive. Proliferation index of the stromal cells was low as compared to tubular cells, as shown by bromodeoxyuridine incorporation.

KEY WORDS: embryonic kidney, neurofilament, stroma

Introduction

The stroma of the embryonic metanephric kidney consists of clear-cyttoplasmic, spindle-shaped cells. In the adult mammalian kidney, this cell type is sparse (Wolgast, 1985), but it is abundant in fetal kidneys (Kissane, 1983). The molecular characteristics and developmental role of the renal stromal cells have remained unresolved. In organ culture, the stromal cells affect the branching morphogenesis of the ureter as well as the tubule-formation of the mesenchymal cells (Unsworth and Grobstein, 1970; Sariola et al., 1988a). Epidermal growth factor promotes cell proliferation of embryonic renal stromal cells (Weller et al., 1991) and prevents their programmed cell death or apoptosis (Koseki et al., 1992; Coles et al., 1993).

The results of viral transfection experiments with cultured kidney rudiments have suggested that the nephrogenic mesenchymal cells are pluripotent with differentiation options toward both epithelial and stromal cell lineages (Herzlinger et al., 1992). In contrast to this are the results from interspecies chick-/quail chimeras and neural crest labeling studies showing that neural crest cells are migrating into metanephric mesenchyme (LeDouarin and Teillet, 1974; Bronner-Fraser and Fraser, 1988). Moreover, early avian metanephric kidney contains adrenomedulla-like cells with catecholamine granules (LeDouarin and Teillet, 1974). A neural crest marker molecule, disialated ganglioside GD3, is expressed in the stromal cells of the mouse embryonic kidney (Sariola et al., 1988a), and cultures of mouse metanephric kidney rudiments contained neural cells (Sariola et al., 1988b). The data suggests that neural-crest-derived cells are present in the embryonic kidney and may have their own developmental bias different from that of other cells of the metanephric mesenchyme. We have analyzed neurofilament proteins and mRNA, as well as the expression of neural cell adhesion molecule L1 of the embryonic metanephric kidneys. Incorporation of bromodeoxyuridine (BrdU) in different cell types was compared to analyze the cell proliferation index of the stromal and epithelial cells.

Neurofilaments are cytoskeletal intermediate filaments that are 10 nm in diameter and several microns long. Three polypeptides of apparent molecular weights of 200 kDa, 160 kDa, and 68 kDa constitute neurofilament heavy (NF-H), medium (NF-M) and light (NF-L) proteins, respectively (reviewed by Liem, 1993). Neurofilaments are found mainly in axons, and during neurogenesis the three subunits are differentially expressed. The NF-M and NF-L proteins are expressed simultaneously during early embryonic development, but the NF-H expression begins at later stages or postnatally (Shaw and Weber, 1982; Julien et al., 1986; Carden et

Abbreviations used in this paper: BrdU, bromodeoxyuridine; NF-L, neurofilament light; NF-M, neurofilament medium; NF-H, neurofilament heavy.
Neurofilaments form the normal radial structure of axons (Friede and Samorajski, 1970; Hoffman et al., 1987; Cleveland et al., 1991; Yamasaki et al., 1992) with NF-L subunit core and NF-M and NF-H coassembly (Delacourte et al., 1980; Geisler and Weber, 1981; Liem and Hutchison, 1982; Hisanaga and Hirokawa, 1988; Troncoso et al., 1990). NF-L is able to self-assemble and form a long intermediate network in axons, but NF-M and NF-H produce only very short filamentous structures (Delacourte et al., 1980; Geisler and Weber, 1981; Liem and Hutchison, 1982; Aebi et al., 1988; Hisanaga and Hirokawa, 1988, 1989; Troncoso et al., 1990; Hains et al., 1993). Although the neurofilaments are characteristic of neuronal cells, they have also been found in certain neural crest-derived and Schwann cells.

We show that the renal stroma includes a spindle-shaped cell lineage that expresses both NF-L and NF-M from the early stage of development on to neonates. Thus, the mesenchyme of the kidney rudiment contains cells with either a tubulogenic or stromal differentiation option, and the stromal cells are characterized by their neural-type intermediate filament structure. The proliferation index of the spindle shaped stromal cells remained very low, showing that during kidney morphogenesis the difference in cell proliferation contributes to the diminishing proportion of the stroma. The histogenesis of the spindle-shaped stromal cells remains open, but the expression of neural characteristics in renal stroma raises the possibility that these cells are of neural crest rather than mesodermal origin.
Results

Both immunohistochemical staining and in situ hybridization revealed an identical pattern of expression for neurofilament proteins and NF-L transcripts. At day 13.5 the embryonic rat kidney rudiment consists of the branching ureter bud and the nephrogenic mesenchyme. NF-L mRNA was seen in a few cells adjacent to the first group of condensing pretubular cells, but not within the condensate (Fig. 1A-C). The transcripts were seen at the medial, caudal and cranial sides of the pretubular condensate, but not lateral to it (Fig. 1B and C). L1 neural cell adhesion protein (L1) is widely expressed in different epithelial and neural cell types (Probstmeier et al., 1990; Nolte and Martini, 1992). Double labeling of the E13 kidney rudiment with an antibody against L1 and neurofilaments (Fig. 1D-F) showed that L1 was expressed not only in the neurofilament-positive microganglion derived neuronal cells, but also in the ureter bud (Fig. 1D). The antibodies against L1 could therefore, conveniently, be used to define the spatial relationship of neurofilament-positive cells with ureter bud-derived epithelial cells. As with in situ hybridization, immunocytochemistry detected neurofilament-positive cells adjacent to the first pretubular condensate (Fig. 1D-F).

At later stages of renal development in situ hybridization revealed expression of NF-L mRNA in cells around the branches of the ureter bud (Fig. 2A-F). These NF-L mRNA-positive peritubular cells did not resemble L1-positive neuronal cells in their morphol-
Fig. 3. Expression of neurofilament proteins around kidney tubules in E18 kidneys. A frozen section double labeled with anti-neurofilament (A) and anti-laminin (B), kidney tubules. Whole-mount preparations of E16 kidneys reveal neurofilament expression (C and E) around the L1 neural cell adhesion molecule-positive collecting tubules (D and F). (C and D) Medullary region; (E and F) the cortical region. Note that neurofilaments are not seen in the uppermost region of the kidney cortex in E. Bars, A-B: 30 μm; C-F: 50 μm.

The peritubular NF-L transcripts were seen in the spindle-shaped and clear-cytoplasmic stromal cells (Fig. 2C and D). Other stromal cells further away from the branches of the ureter bud were negative for NF-L transcripts, but by morphological criteria they resembled the NF-L mRNA-positive peritubular cells (Fig. 2A-C).

The expression of neurofilaments in the peritubular stromal cells in the E16 metanephric kidneys is shown in Fig. 3. Whole-mount immunolabeling of embryonic kidneys provides a good "three-dimensional" view of different structures. In addition to the stromal expression of neurofilaments (Fig. 3C-F), such explants revealed typical neuronal cells with neurites in a microganglia-like structure that was located adjacent to the stalk of the ureter (Fig. 4). This group of cells could also be seen by in situ hybridization in the hilus of the kidney (Fig. 4A).

A clear molecular difference between the neurofilament-positive stromal cells and the microganglion neuronal cells could be demonstrated by double-immunolabeling the whole-mount explants with the polyclonal antibody against L1 neural cell adhesion protein...
assay indicates that the cell proliferation is high both in the condensing pretubular mesenchyme in the S-shaped epithelia, and in the cortical branches of the ureter bud, but low in the renal stroma.

Discussion

The present study was undertaken to analyze molecular features and proliferation characteristics of the spindle-shaped, clear cytoplasmic stromal cells of the embryonic kidney. Unexpectedly, NF-L and NF-M proteins, and NF-L transcripts were found not only in the cells of the renal microganglion, but also in the cells of the peritubular stroma. As compared to the epithelial cells, these cells showed a low rate of BrdU incorporation, which suggests that low cell proliferation may contribute to the diminishing proportion of the stroma during late kidney morphogenesis.

Two-step induction of kidney morphogenesis

Kidney development is an example of organogenesis guided by heterotypic interactions between several cell lineages which differentiate and assemble in a synchronized manner. The interaction between the ureter bud and the mesenchymal blastema leads to a series of sequential metabolic and morphological changes (Saxén et al., 1968; Ekblom et al., 1981; Saxén, 1987). A variety of heterotypic tissues can trigger the tubulogenesis and lead to formation of typical kidney tubules (Grobstein, 1955; Lombard and Grobstein, 1969; Unsworth and Grobstein, 1970). Before the ureter bud enters the blastema, the nephrogenic mesenchyme has developed a “tubulogenic bias” as no other embryonic mesenchyme can be converted into tubules of the secretory nephron (Saxén, 1970). After invasion of the ureter bud, the tubulogenic cells have initially rescued from apoptosis (Koseki et al., 1992). The surviving, “induced” cells are then stimulated to proliferate, they become aggregated and ultimately convert into epithelial structures.

Increasing intercellular adhesion leading to aggregation of the induced cells is a prerequisite for their further differentiation and polarization (Saxén et al., 1988). It has been suggested that this stage is not affected primarily by the ureter bud, but that a two-step

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mechanism operates during tubulogenesis (Gossens and Unsworth, 1972). Initially the contact with the ureter bud might lead to induction of certain key molecules, such as WT-1 and pax-2, that selectively activate the genes required for further nephrogenesis and repress the others (Dressler and Douglass, 1992; van Heyningen and Hastie, 1992; Kreidberg et al., 1993). The subsequent second stage of induction would be regulated by an interaction between the induced pretubular cells and the surrounding stroma. In accordance with this proposal, an antibody against the cell surface ganglioside GD₃, an antigen expressed in the stroma of the embryonic mouse metanephros, perturbs nephrogenesis (Sariola et al., 1989). The two-step induction hypothesis provides a possible explanation for coordinated and controlled complex morphogenesis of the cell lineages taking part in the various functions of a mature nephron. Only the nephrons in a proper local environment (i.e. neurons, blood vessels, stroma etc.) continue their differentiation, while the others cease to differentiate and undergo apoptosis.

The developmental fate of the embryonic renal stroma

The developmental fate of the embryonic renal stroma is unknown, but it represents only a minor population in adult kidneys. Either the peritubular cells have a low proliferation rate or they undergo apoptosis. Koseki et al. (1992) described apoptosis in peritubular stromal cells. These apoptotic cells apparently represent the stromal cells with neuronal characteristics. Even though precise calculations are not available, most of the apoptosis in the embryonic kidney seems to occur in the stroma (Koseki et al., 1992). Coles et al. (1993) recently estimated that 50% of the metanephric cells die through apoptosis during kidney differentiation. We now show by the BrdU incorporation assay that the proliferation index in the stromal cells is much lower than in epithelial cells or in the induced condensates. This changes the ratio of stromal versus epithelial cells during morphogenesis and will ultimately result in epithelial predominance in neonatal kidney, even without apoptosis of the stromal cells. Thus, both a low proliferation index and apoptosis of stromal cells lead to the paucity of stroma in adult metanephros.

L1 neural cell adhesion molecule in epithelial collecting ducts and neurons

L1 neural cell adhesion molecule (Rathjen and Schachner, 1984) has been detected in several neural and non-neural cell types (Probstmeier et al., 1990; Nolte and Martini, 1992). It affects neuronal outgrowth through both homophilic and heterophilic association of cell adhesion molecules (Sonderegger and Rathjen, 1992). We now show that L1 is expressed on embryonic collecting tubules and renal neurons. Of the two different renal cell types with neurofilaments, L1 is expressed only on the typical neuronal cells, but not on the stromal cells. The practical consequence of this finding is that immunocytochemistry can be used in defining these two cell lineages. It also indicates that, if L1 is involved in the pattern formation of the stromal cells or in the heterophilic-mesenchymal interaction, it acts through the heterophilic rather than homophilic binding principle.

The developmental origin of the renal stroma

The transplantation studies with chick-quail chimeras (LeDouarin and Teillet, 1974) and neural crest labeling with a vital dye (Bronner-Fraser and Fraser, 1988) have traced trunk neural crest cells to mesonephric and metanephric kidney. The neural crest gives rise to numerous neuronal and non-neuronal phenotypes, such as peripheral neurons, Schwann and glial cells, pigment cells, head mesenchyme, and cartilage (reviewed by LeDouarin, 1982). Our present observations show that the spindle-shaped stroma of the embryonic kidney includes cells that possess neuronal characteristics. The unexpected neuronal feature of the stromal cells raise the possibility that they may be the neural crest derivatives in the embryonic metanephric kidney, a hypothesis that deserves further experimental studies.

Materials and Methods

Animals

Sprague-Dawley rat and CBaxNMR mouse embryos were used throughout the study. Animals were mated overnight (E0). In mice, the appearance of the vaginal plug was considered as day 0. Embryonic rat kidneys were microsurgically dissected at different stages beginning from E13, and the stage of the embryos was further verified by the size of the ureter bud.

Antibodies

The mouse monoclonal antibody 13AAB (diluted 1:2 in phosphate buffered saline: PBS) against purified bovine neurofilament polypeptides (Virtanen et al., 1985) reacts with all three neurofilament proteins of mouse cerebellum (Tienari et al., 1987). This antibody was used in double labeling experiments with different polyclonal antibodies. The different subunits of the neurofilament triplet were analyzed in the whole-mount preparations with monoclonal antibodies detecting specifically either NF-L, NF-M, or NF-H (Boehringer Mannheim, Germany). These three antibodies were used at 1:10 dilution. The rabbit polyclonal affinity purified antibody against mouse brain neural cell adhesion molecule L1 (Rathjen and Schachner, 1984) was diluted 1:100. Rabbit polyclonal antibody (diluted 1:400 in PBS) against mouse EHS-laminin was from Gibco-BRL (Paisley, Scotland). The secondary rhodamine-conjugated goat anti-mouse IgG (dilution 1:100) and the fluorescein-conjugated donkey anti-rabbit IgG (dilution 1:200) antibodies were from Jackson Immunoresearch (West Grove, PA, USA).

Immunohistochemistry

The dissected kidneys were fixed with 20% methanol for 5 min at room temperature and rinsed with PBS containing 11.6% sucrose and 1% BSA, and stained by whole-mount immunofluorescence as previously described (Sariola et al., 1988). Kidneys of different developmental stages were also dissected for frozen section immunohistochemistry, mounted in Tissue Tek O.C.T. compound (Ekhard, IN, USA), frozen in dry ice and sectioned at 6 μm. Sections were fixed with 20% methanol for 5 min or acetone for 10 min, rinsed with PBS, and double-stained with different antibodies against tubular and neuronal antigens for 1 h or over night. Sections were stained with secondary antibodies for 1 h and mounted with Elvanol.

Construction of the probes

The cDNA probe encoding for the neurofilament light (NF-L) protein was subcloned into pGEM™-4Z vector from the full-length rat NF-L cDNA (J. Pali, unpublished results; probes were purchased from Promega). Rat 35S-UTP-labeled sense and antisense cRNA probes were prepared and hybridized as described (Wilkinson and Green, 1990).

In situ hybridization

E13-15 whole embryos and kidneys from E13-21 embryos were fixed in phosphate-buffered 4% paraformaldehyde overnight at 4°C or 1-2 h at room temperature, respectively. Adult rat kidneys were perfusion fixed with the same fixative. The tissues were embedded in paraffin, and serial sections were cut at 5 μm on triethoxysilane- and acetone-treated slides. The 35S-UTP-labeled sense and antisense cRNA probes were prepared and the in situ hybridization was performed according to Wilkinson and Green (1990). The probes were diluted to the final concentration of 20×10⁶
Bromodeoxyuridine Incorporation

BrdU was injected intraperitoneally to mice at day 16 of pregnancy. After 2 h the mice were sacrificed and the embryos were fixed in Bouins fixative, embedded in paraffin, and serially sectioned at the level of the kidneys. The incorporated BrdU was analyzed by indirect immunoperoxidas staining with anti-BrdU antibody and Vectastain ABC-kit (Vector Laboratories, Burlingame, CA, USA), and the proliferation index was counted as the ratio of labeled and unlabeled nuclei. BrdU label and the mouse anti-5-bromo-2'-deoxyuridine antibody were from Amersham (Buckinghamshire, UK).

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