Early innervation of the metanephric kidney

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Summary

During kidney differentiation, the nephrogenic mesenchyme converts into renal tubules and the ureter bud branches to form the collecting system. Here we show that in the early undifferentiated kidney rudiment there is a third cell type present. In whole-mount preparations of cultured undifferentiated metanephric kidneys, neurones can be detected by immunohistochemical means with antibodies against the neurofilament triplet, 13AA8, and against neuronal cell surface gangliosides, Q211. Clusters of neuronal cell bodies can be seen in the mesenchyme close to the ureter bud. The terminal endings of neurites are found around the mesenchymal condensates that later become kidney tubules. A similar distribution of neurites can be revealed in tissue sections of kidney grafts growing in the chicken chorioallantoic membranes. In primary cultures of the ureter bud cells, neurones are constantly present. In another report, we have shown that, in experimental conditions, neurones are involved in regulation of kidney morphogenesis. The present results raise the possibility that neurones of the metanephric kidney may have this function in vivo as well.

Key words: kidney differentiation, neuronal differentiation, kidney innervation, morphogenetic interaction.

Introduction

The embryonic metanephric kidney serves as a classical model for morphogenetic tissue interactions. The early kidney rudiment is composed of the nephrogenic mesenchyme and the ureter bud, which is an evagination of the Wolffian duct (for a comprehensive review, see Saxén, 1987). Isolation experiments have shown that, when cultured separately, the isolated mesenchyme does not differentiate nor does the ureter bud branch (Grobstein, 1955). However, when the mesenchyme is recombined with the ureter bud or with some other embryonic tissues, tubular differentiation proceeds (Grobstein, 1955a, 1955). No other embryonic tissues can be converted into kidney tubules (Saxén, 1970). Hence, only the unique nephrogenic mesenchyme has 'a kidney bias'. It has been concluded that the mesenchyme is predetermined for nephrogenesis and, therefore, the influence of the inductor tissue on the mesenchyme is permissive in nature (Saxén, 1977). The molecular background of the epitheliomesenchymal tissue interactions is not yet understood (see Gurdon, 1987). Perturbation experiments with antibodies against the cell adhesion molecule N-CAM (Klein et al. 1988) and uvomorulin (Vestweber et al. 1985) showed that those molecules were not involved in inductive events although they were expressed either on mesenchymal or epithelial cells.

Since several embryonic tissues trigger differentiation of the nephrogenic mesenchyme (Grobstein, 1955; Unsworth & Grobstein, 1970; Lombard & Grobstein, 1969; Auerbach, 1972), we have recently further evaluated the cell-type specificity of kidney induction. Separated undifferentiated nephrogenic mesenchymes were combined with cultures of chicken tectum cells that effectively promoted tubulogenesis. Lysis of neurones prevented induction, suggesting that, in these experiments with a heterologous inductor, neurones were required for induction of tubular differentiation (Sariola et al. 1989). By analysing tissue sections, we did not find neurones in early embryonic kidneys. Therefore, it was not clear whether or not neurones might be involved in regulation of normal kidney differentiation.

In the present paper, we demonstrate a technique
with which we detect neurones in cultures of embryonic kidneys and can localize neurites in the areas of tubule induction. Hence, the results raise the possibility that neurones may induce nephrogenesis in vivo.

**Materials and methods**

**Tissue culture and grafting experiments**

Undifferentiated and avascular murine (CBA×NMRI) kidneys were microdissected from 11-day-old embryos and cultured either in vitro in a Trowell-type culture in Dulbecco’s minimum essential medium supplemented with 10% fetal calf serum or grafted on 7-day-old chicken chorioallantoic membranes. The kidneys were cultured in vitro for 1 to 5 days and in the chorioallantoic membranes up to 10 days. Details of the culture systems have been published previously (Grobstein, 1953a; Saxén et al. 1968; Ekblom et al. 1982). Briefly, the kidneys were cultivated in vitro on 1-0.um pore-size Nuclepore filters (Pleasanton, California, USA) placed on a metal grid. For transplantation experiments a hole was cut into a 7-day-old egg, the shell membrane was carefully scraped off and the kidneys were pipetted on the chorioallantoic membrane. The egg was sealed with a piece of Scotch tape and incubated thereafter at 37°C (Sariola et al. 1983). For each analysis of neuronal elements (see below) at least 10 kidney explants or grafts were used.

**Immunohistology**

**Antibodies and fixation**

The neurone cell-surface-specific mouse monoclonal antibody Q211 reveals tetra- and pentasialated gangliosides (Henke-Fahle, 1983; Rössner et al. 1985). Another mouse monoclonal antibody, 13AA8, recognizes in mouse all the three neurofilaments (Virtanen et al. 1985; Tienari et al. 1987). It was a generous gift of Dr Ismo Virtanen (University of Helsinki, Finland). The polyclonal rabbit antibody against laminin was purchased from Bethesda Research Laboratories (Maryland, USA). The rabbit antibody against fibronectin (Sariola et al. 1984) was a generous gift of Dr Pentti Kuusela (University of Helsinki, Finland). The dilutions for immunohistochemical staining were as follows: 13AA8, 1:5; Q211, 1:2000; α-fibronectin and α-laminin antibody, 1:300.

For staining with Q211, the tissues were fixed for 20 min in 3.5% paraformaldehyde. For staining with α-laminin, α-fibronectin and 13AA8 antibodies, the tissues were fixed for 10 min in cold (−20°C) methanol. The methanol fixation permeabilizes the tissue, whereas after paraformaldehyde fixation the antibody penetration into the tissue is poor. Since gangliosides are dissolved by methanol, it could not be used for staining with Q211. Therefore, in the paraformaldehyde-fixed material stained by the antibody Q211, only neurones that are located on or close to the surface of the kidney are detected, whereas after methanol fixation, the antibody 13AA8 reacts with the neurofilament-containing neurones also within the kidney explant. The penetration of the antibodies could be confirmed and the nphric tubules visualized by double labelling the tissue with the polyclonal antibody against laminin.

The second antibodies were fluorescein isothiocyanate (FITC)-conjugated antibody against mouse immunoglobulins and TRITC-conjugated antibody against rabbit immunoglobulins (Cappel, West Chester, USA). The second antibodies did not show any species cross-reactivity and could, therefore, be used in double-labelling experiments. When the primary antibody was omitted or O-serum was used in its place, no staining was seen.

**Tissue sections**

The tissues were frozen with dry ice, cut at 10 μm and stored at −20°C. All tissue sections were incubated overnight in the primary antibody, washed three times for 10 min with phosphate-buffered saline (PBS), incubated for 30 min in the secondary antibody, washed three times for 10 min in PBS, mounted with Elvanol (Hoechst, Darmstadt, FRG) and viewed on a Leitz Axioptot fluorescence microscope with epi-illumination and appropriate filters.

**Whole mounts**

Cultured whole kidneys were stained by a slightly modified indirect immunofluorescence method with prolonged incubation times. The whole tissue explants (‘whole mounts’) attached to the Nuclepore filter were incubated for 2 days in the primary antibody, washed three times for 2 h each in PBS, followed by an overnight incubation in the secondary antibody, three washes for 2 h in PBS and mounting in Elvanol. All incubations were done in a humidified chamber at 4°C. In double-labelling experiments, the same procedure was repeated consecutively with appropriate antibody combinations.

**Results**

**Tissue culture experiments**

Our recent immunofluorescence studies of embryonic kidneys had shown that by using the conventional immunofluorescence technique of first cutting and then staining the tissue sections, the pattern of neurones is very difficult to evaluate (Sariola et al. 1989). Therefore, 11-day-old undifferentiated whole kidneys were first cultured in vitro, stained in toto after 6 h to 5 days of culture and subsequently analysed by the indirect immunofluorescence technique.

Both Q211 and 13AA8 antibodies revealed neuronal cell bodies and neurites in these whole-mount cultures. Neurofilament expression was seen after one day of culture and thereafter (Fig. 1). In 4- to 5-day-old cultures, two to three clusters of neurones, ‘microganglions’, were seen in the periphery of the tissue. The ganglion beneath the Wolffian duct was constantly found, whereas the number of ganglions in the peripheral area varied from 0 to 2 (Figs 2 and 3). Within the kidney, few neuronal cell bodies were seen, one or none around the tips of the ureter bud.
However, the neurites from the Wolffian duct ganglion grew into the stromal areas with ramifying neuritic extensions around the cell condensates that surround the tips of the ureter bud (Fig. 4). These cell condensates later form secretory tubules of nephrons.

When the nonpermeabilizing paraformaldehyde fixation was used, the antibody Q211 revealed only neurones that are located on the surface or close to it. Q211 binding was first seen after two days of culture. Also with Q211, neurites were seen to end up in the
Fig. 2. Expression of neurofilaments (the left panel) and laminin (the right panel) after 4 to 5 days of culture of undifferentiated kidneys. (A) At day 4, the nerve fascicles become thicker. However, because of the thickening of the explant, thin neurites are now difficult to visualize by low magnification. (B) Double label with α-laminin antibody shows that the ureter bud has undergone several tertiary branchings. (C) Day 5. In this explant two clusters of neuronal cells are seen in the periphery of the culture. This finding is not such a constant feature like the presence of neurones in the mesenchyme around the stalk of the ureter. Peripheral microganglions were seen only in 14 out of the 23 kidney cultures. (D) Double label with the α-laminin antibody. Bar, 500 μm.
Early innervation of the kidney

The peripheral area of the explant, close to the tips of the ureter bud (Fig. 5). However, the number of labelled neurones was smaller with the Q211 antibody than with 13AA8.

Kidney grafts on chorioallantoic membranes

When undifferentiated mouse kidneys are transplanted on chicken chorioallantoic membranes (CAM), they differentiate and are vascularized by the chicken vessels (Ekblom et al. 1982; Sariola et al. 1983). In the chimeric kidney grafts, incubation of sections with Q211 and 13AA8 did not reveal any staining of the CAM itself. Within the graft, neurones were found in the stromal compartment. A cluster of neuronal cell bodies was seen around the stalk of the ureter (Fig. 6A and B). In the cortical areas of the kidney grafts, the neurites spread and were seen around the induced cell condensates (Fig. 6C and D).

Separation experiments

The ureter bud can be microsurgically dissected from the kidney mesenchyme (Grobstein, 1955; Saxén et al. 1968) and cultured in vitro. However, the separated tissues do not differentiate in isolation and flatten out. When grown on cover slips, the isolated mesenchymes soon degenerate, but the ureter buds can be cultivated for at least 7 days. In 3- to 7-day-old primary cultures of the ureter bud, 13AA8 and Q211 labelled neurones growing on the epithelial cells (Fig. 7). This finding was constant, since neurones were found in all 20 ureter bud cultures tested for presence of neurones.

Discussion

Our in ovo and in vitro analyses of embryonic kidneys show that, in the undifferentiated metanephric kidney, neuronal cell bodies are present. The separation experiments and the analysis of kidney cultures suggest that axonal extensions spread in the stromal area and along the surface of the ureter bud. Their invasion into the nephrogenic tissue precedes angiogenesis and morphological differentiation of the mesenchyme. When undifferentiated kidneys are cultured in vitro, blood vessels are normally not present (for review, see Sariola, 1985), and yet axonal

Fig. 3. High magnification of the area around the stalk of the ureter (2-day culture shown at low magnification in Fig. 1A and B). (A) When focused on the top of the explant, a cluster of neuronal cell bodies is seen. (B) Deep in the tissue, the neurites surround the ureter bud and the condensed mesenchyme. (C) The α-laminin staining of the same area. u, ureter bud; cm, condensed mesenchyme; t, tip of the ureter bud. Bar, 100 μm.
Early innervation of the kidney

Fig. 5. Q211 antibody reacts with polysialated gangliosides and, therefore, paraformaldehyde fixation was used instead of methanol which dissolves gangliosides. In these whole mounts, only the superficial neurones become visible. (A) 3-day culture of an 11-day-old kidney. Although the number of stained neurites is minute with Q211, several neurites are seen above the areas where the mesenchyme condenses around the tips of the ureter. (B) The phase-contrast view shows the tip of the ureter bud. u, ureter bud; cm, condensed mesenchyme. Bar, 1250μm.

Fig. 4. High magnification of a peripheral area of a kidney explant cultured for 3 days. The left panel is a α-neurofilament and the right panel α-laminin staining. (A) When focused deep in the explant, some neurites are seen close to the stalk of the ureter. (B) Laminin expression reveals the basement membrane around the ureter. (C) More superficially in the same area, the tips of the ureter turn up and right. Two tips are seen at this focusing level. Neurites are seen in and around the mesenchyme around the tips, but they do not attach to peripheral endings of the branches. (D) The double label with the α-laminin antibody. u, ureter; t1 and t2, tips of the ureter bud. Bar, 1250μm.

elongation occurs. Hence, innervation and angiogenesis may be spatially related, but are clearly independent and separable developmental events.

Several histochemical studies have found neurones in the kidney (for a review, see Asfoury, 1971), but innervation has not previously been analysed in the undifferentiated kidney. In adults, the renal nerves can be basically divided into extrinsic and intrinsic ones. The present results demonstrate that the intrinsic neurones invade the embryonic kidney early in development. This is in accordance with the observations on chick–quail neural crest chimeras. LeDouarin & Teillet (1974) found quail-derived crest cells in the stroma of chicken kidneys, when a piece of the chicken neural crest had been replaced by quail neural crest. At that time, it was considered to be an experimental artefact. Recent publications show that afferent nerve endings of renal nerves can be found in the brain stem (Cirello & Calares, 1983; Wyss & Donovan, 1984). Since programmed cell death of embryonic neurones is a major feature in the development of the nervous system, it is possible, however, that the early embryonic neurones are not the same ones as in the adult kidney. Furthermore, it is not yet known whether the neurones of the kidney rudiment are all similar or contain different neurotransmitters.

Biochemical and immunohistochemical analyses have shown that the adult kidney contains a diversified pattern of neurotransmitters, including epinephrin, norepinephrin, acetylcholine, substance P and dopamine (Dinerstein et al. 1983; Kuo et al. 1984; Barajas & Wang, 1983; Ballesta et al. 1984; Ferguson & Bell, 1985; Morgunov & Baines, 1985; Summers et al. 1985). The nerves regulate both renal circulation as well as secretory functions (Mancia & Zanchetti, 1979; Torretti, 1982; Gullner, 1983; Imbs et al. 1984; Karlberg, 1983; DiBona, 1985; Koepeke & DiBona, 1985). Nothing is known about their functions during normal embryogenesis.
The studies by Grobstein (1955) have shown that, in experimental conditions, kidney differentiation can be induced with brain tissue. He microdissected the nephrogenic mesenchyme and recombined it with different embryonic tissues. Some of them, like heart and lung, did not induce tubular differentiation,
Early innervation of the kidney

whereas others, like spinal cord, brain, salivary gland, head and jaw mesenchyme, effectively induced nephrogenesis (Grobstein, 1955; Auerbach & Grobstein, 1958; Lombard & Grobstein, 1969; Unsworth & Grobstein, 1970). Transfilter experiments (Grobstein, 1953b, 1956), in which the nephrogenic mesenchyme and the inducing brain tissue are glued on opposite sites of a porous filter, have indicated that close cell-to-cell contacts are required for tubule induction (Wartiovaara et al. 1972; Lehtonen, 1976). In mouse, spinal cord material grows rapidly, within 1 h, through 1 μm pores, but does not pass through 0.05 μm pores (Lehtonen et al. 1975; Saxén & Lehtonen, 1978). Surprisingly, only spinal cord, but not the other inducers defined recombinant cultures, effectively induces tubulogenesis in transfilter cultures (Sariola et al. 1989). Immunohistochemical studies showed that neurites from spinal cord penetrate through the mesenchyme in 16 h. This is also the minimum time after which the spinal cord can be scraped off and tubular differentiation then proceeds in the absence of an inductor tissue (Nordling et al. 1971; Lehtonen et al. 1983). It may be that although cell processes from all inducer and non-inducer tissues grow through 1 μm filter-pores, in transfilter cultures only spinal cord can establish contacts deep enough within the mesenchyme to trigger morphogenesis.

Another possible explanation was evaluated by analysing the cell-type specificity of induction within the inductor tissue. This issue was studied with chicken tectum cell cultures that turned out to be potent inductors (Sariola et al. 1989). However, when neurones were lysed by means of complement and the neurone-specific antibody Q211, no tubules formed. Hence, the results suggested that in these cultures neurones were required for induction of tubulogenesis.

At that time, we did not find neurones in the undifferentiated embryonic kidney. Their presence can now, however, be demonstrated in the in vitro and in ovo cultures of microdissected kidney rudiments with antibodies against neurofilaments and cell surface gangliosides. Because neurites are thin and extremely long in these cultures, the whole-mount technique turned out to be more sensitive and reliable than tissue sections in judging the pattern and distribution of neurones. In the central nervous system, neurofilaments and multisialated gangliosides appear late in embryogenesis and serve as markers for terminal differentiation of neurones (Tapscott et al. 1981; Rösner et al. 1985). In the early mouse kidney, both neurofilament and Q211 stainings were negative suggesting that, initially, the neurones have migrated into the kidney as neuroblasts. This supports the finding that some neuroblasts leave the neural tube or

Fig. 7. Primary culture of an isolated ureter bud cultivated for 2 days on a glass cover slip. The microsurgically dissected ureter bud consists of an epithelial bud surrounded by a sheet of loose mesenchymal cells. (A) Neurofilament staining reveals neurones in the mesenchyme around to the isolated ureter bud. Some neurites grow attached to a monolayer of cells around the explant. (B) Double label with α-laminin antibody shows that both the compact cell cluster and the surrounding monolayer of cells express laminin. Bar, 1000 μm.
the neural crest as undifferentiated, still proliferating, cells (for review, see Watterson, 1965).

The present data raise the possibility that the early nerves of the embryonic kidney might be involved in regulation of morphogenesis. The localization of terminal endings of neurites around the condensates of the mesenchyme fits well into this hypothesis. Although we cannot exclude the possibility that some neuroblasts could be found within the undifferentiated mesenchyme, the primary culture experiments with the ureter bud and the Wolffian duct suggest that at least some neurones are initially situated in the mesenchyme close to the ureter bud and grow along its wall. In the microsurgical separation and recombination experiments that suggested a role for the ureter bud in kidney differentiation (Grobstein, 1955), these neurones may have been dissected from the mesenchyme with the ureter bud. On the other hand, the neurones and the ureter bud may act on the kidney mesenchyme as an inductor unit, in which both the neuronal and non-neuronal cell types have a specific function in tubule induction. This kind of a multistep induction mechanism has been proposed from primary induction (Saxén et al. 1964; Saxén & Toivonen, 1961). In the metanephric kidney, the ureter bud may be responsible for the initial condensation of the mesenchyme and the neurones for triggering of differentiation. Further experiments, however, are required to characterize the nature of the embryonal neurones in the kidney, and to give direct experimental evidence for the present observations.

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