Expression of epidermal growth factor in the developing rat kidney

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EPIDERMAL GROWTH FACTOR (EGF) is a 53-amino acid polypeptide that has been isolated from the mouse salivary gland and characterized by Cohen (6). EGF mRNA has been identified in mammalian kidney tissue, and EGF is present in relatively high concentration in urine (22). EGF and EGF mRNA levels become detectable in rodents during the neonatal period. The role of EGF in the developing kidney is unclear. However, a mitogenic role for tubular cells has been proposed. EGF is a potent mitogenic factor for the proximal tubule epithelium and appears to function as a survival factor in renal cells in the developmentally mature kidney by preventing apoptosis (15). EGF also induces branching of the isolated ureteric bud and stimulates the morphogenesis of the collecting duct (14, 30). It also plays an important role in tubular cystic formation, tubular hyperplasia, and segmental differentiation resulting from the tyrosine kinase activity of the EGF receptor during renal development (8, 11, 18, 26). Gattone et al. (12) reported that EGF immunoreactivity was first detected in the developing mouse distal nephron at day 6 after birth (P). A previous study from our laboratory demonstrated that numerous S5′-bromo-2′-deoxyuridine (BrdU)-positive cells were found in SHT1α-positive thick ascending limb (TAL) of the outer medulla and medullary rays at P3 and P7 (5). The TAL is one of main sites of cell proliferation and differentiation in the developing kidney. However, it is unknown whether the role of EGF is for proliferation during tubule maturation. While it is well established in adult animals that EGF is expressed in both the thick ascending limb and the distal convoluted tubule, little is known about the expression and the precise distribution of EGF in the developing rat kidney and to identify which cells are EGF positive and which EGF negative in the developing loop of Henle. Our study was therefore designed to establish the time of expression and the pattern of distribution of EGF in the developing rat kidney and to identify which cells are EGF positive and which EGF negative in the developing loop of Henle.

MATERIALS AND METHODS

Animals and BrdU Treatment

Sprague-Dawley rats were used in all experiments. They were the progeny of rats obtained from the Experimental Animal Center of Hallym University. The animals were provided with a commercial diet and water ad libitum under temperature-, humidity-, and lighting-controlled conditions (22 ± 2°C, 55 ± 5%, and a 12:12-h light-dark cycle, respectively). Procedures involving animals and their care were conducted in accordance with our institutional guidelines that comply with international laws and policies [Guide for the Care and Use of Laboratory Animals (NIH Publication 85–23, 1985)].

The kidneys were obtained from 18 (E18)- and 20-day-old (E20) fetuses, 1 (P1), 3 (P3), 7 (P7), 14 (P14)-, and 21-day-old (P21) pups, and adults. The proliferation rate of EGF-labeled cells was measured by administering a single injection of 50 μg/g body wt of BrdU (Boehringer Mannheim, Mannheim, Germany) to P3 and P7 animals 18 h before death; the kidneys were subsequently preserved for immunohistochemistry. BrdU is a thymidine analog incorporated into DNA during the S phase of the cell cycle and can be subsequently detected in tissue sections with specific antibodies raised against it (29).

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Tissue Preservation

The animals were anesthetized with an intraperitoneal injection of pentobarbital sodium (50 mg/kg body wt). The kidneys were preserved by in vivo perfusion of the cardiac or abdominal aorta. The animals were initially perfused with PBS (osmolality 298 mosmol/kg H2O, pH 7.4) to wash out the blood. This was followed by perfusion with a periodate-lysine-2% paraformaldehyde (PLP) solution for 5 min. After perfusion, the kidneys were removed and cut into 1- to 2-mm-thick slices that were further fixed by immersion in PLP solution overnight at 4°C. Sections for immunoperoxidase preembedding staining were cut transversely through the entire kidney on a Pelco 101, Sectioning Series Vibratome (Technical Products International, St. Louis, MO) at a thickness of 50 μm and processed for immunohistochemical studies by use of a horseradish peroxidase preembedding technique.

Antibodies

EGF immunoreactivity was detected using an affinity-purified rabbit polyclonal antibody against EGF (Biosource Technologies, Vacaville, CA). The TAL was identified by use of rabbit polyclonal antibody against the Na-K-2Cl cotransporter (NKCC2, BSC-1; courtesy of Dr. Mark A. Knepper, National Institutes of Health). The antibody labels the apical plasma membrane of thick ascending limb (10). The macula densa of the loop of Henle (MD) was identified by use of rabbit polyclonal antibody raised against a neuronal nitric oxide synthase (nNOS; Sigma, St. Louis, MO). Expression of nNOS in the rat kidney has been studied in detail in previous studies (3, 4). The distal convoluted tubule (DCT) was identified by use of thiazide-sensitive Na-Cl cotransporter (TSC; courtesy of Dr. Mark A. Knepper). The antibody labels the apical membrane of all DCT segments including DCT1 and DCT2 (16). Calbindin D28k (CaBP; Sigma) was also used for DCT2 and the connecting tubule (CNT) segment (20, 27). For the detection of BrdU, a mouse monoclonal antibody against BrdU (Boehringer Mannheim) was used.

Immunolabeling of EGF

Fifty-micrometer Vibratome sections were processed for immunohistochemistry using an indirect preembedding immunoperoxidase method. All sections were washed with 50 mM NH4Cl in PBS three times for 15 min. Before incubation with the primary antibody, the sections were pretreated with PBS containing 1% BSA, 0.05% saponin, and 0.2% gelatin (solution A) for 3 h. The tissue sections were then incubated overnight at 4°C with antibodies to EGF (1: 250) diluted in 1% BSA in PBS (solution B). Control incubations were made using solution B without the primary antibody. After three washes in solution A, the sections were incubated for 2 h in peroxidase-conjugated donkey anti-goat or anti-rabbit IgG Fab fragment (Jackson ImmunoResearch Laboratories, West Grove, PA) diluted 1:100 in solution B. The tissues were then rinsed, first in solution A and then in 0.05 M Tris buffer (pH 7.6). Horseradish peroxidase was detected by incubating the sections in 0.1% 3,3′-diaminobenzidine (DAB) in 0.05 M Tris buffer for 5 min, after which H2O2 was added, to a final concentration of 0.01%, and the incubation was continued for 10 min. After washing with 0.05 M Tris buffer, the sections were dehydrated through a graded series of ethanol and embedded in poly/Bed 812 resin (Polyscience, Warrington, PA). The sections were examined and photographed with a photomicroscope (Olympus).

Immunolabeling of BrdU

Flat preembedded 50-μm-thick Vibratome sections of kidney were processed for double-labeling of EGF and BrdU, or NKCC2 and BrdU...
in the TAL of the loop of Henle. Portions of the cortex and outer medulla were excised and glued onto empty blocks of Epon 812. One-micrometer-thick sections were cut and treated for 10–15 min with a saturated solution of sodium hydroxide, diluted 1:3 in absolute ethanol, to remove the resin. After three brief rinses in absolute ethanol, the sections were hydrated through graded ethanols and rinsed in tap water. To improve DNA accessibility, the sections were treated with 20 μg/ml proteinase K (Fisher Biotech) containing 10 mM ethylenediaminetetraacetate and 10 mM NaCl in 0.05 M Tris buffer (pH 7.8) in a humidified chamber for 5 min at room temperature. This was followed by treatment with 0.2% glycine for 5 min to inhibit the enzyme. After being rinsed in tap water for 10 min, the sections were incubated for 30 min with H₂O₂ in methanol, rinsed again in tap water for 10 min, and treated with 0.5% Triton X-100 in PBS for 15 min. The sections were then rinsed in PBS three times for 10 min before being treated with 1% BSA for 1 h. The sections were incubated with mouse anti-BrdU antibody (1:60) at 4°C. After being washed in PBS, the sections were incubated for 2 h in a mixture of peroxidase-conjugated goat anti-rabbit IgG and peroxidase-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories). Vector SG (Vector Laboratories, Burlingame, CA) was used as the chromogen to detect the peroxidase; this gives a gray blue color, which is easily distinguished from the brown staining produced by DAB used in the preembedding procedure for detection of EGF and NKCC2. The sections were washed with distilled water, dehydrated through graded ethanol and xylene, mounted in Canada balsam, and examined by light microscopy.

Transmission Electron Microscopy

Electron microscopic observations were made on Vibratome sections postfixed with 1% glutaraldehyde and 1% osmium tetroxide in 0.1 M phosphate buffer (pH 7.4) before being dehydrated and embedded in poly/Bed 812 resin. Ultrathin sections were stained with uranyl acetate and lead citrate and photographed using a transmission electron microscope (JEOL 1200EX, Tokyo, Japan).

Immunohistochemistry for Identification of the Loop of Henle

The PLP-fixed kidneys were dehydrated through a graded series of ethanol and embedded in wax (polyethylene glycol 400 disterate, Polysciences). The wax-embedded tissue was cut serially at 2 μm on a rotary microtome (Leica), and the sections were dewaxed and rehydrated. The sections were then rinsed with PBS, incubated in normal donkey serum (Jackson ImmunoResearch Laboratories) for 1 h, and incubated overnight at 4°C with each antibody (1:200 NKCC2; 1:200 nNOS; 1:10,000 TSC; 1:3,000; 1:5,000 CaBP). The sections were then rinsed with PBS, and incubated for 2 h with biotinylated secondary antibody, then with Vectastain ABC reagent for 2 h. The sections were rinsed with PBS, incubated with the peroxidase substrate solution, a mixture of 0.05% DAB and 0.01% H₂O₂, for 2 min at room temperature. The sections were finally rinsed with Tris-HCl buffer, dehydrated through graded ethanol, cleared in xylene, and mounted in Canada balsam for light microscopic examination.
Quantitation of BrdU-Positive Cells

TAL cells, identified by NKCC2, were counted in 1-μm-thick (semithin) sections of the cortex and outer medulla from P3 and P7 animals and expressed as the percentage of the total number of NKCC2-positive cells containing BrdU-labeled nuclei. In other sections, EGF-positive cells with BrdU-labeled nuclei were counted. The values are presented as means ± SD from five animals at P3 and P7 after birth. All data obtained from the quantitative measurements were analyzed using one-way ANOVA to determine statistical significance. Bonferroni’s test was used for post hoc comparisons. A P value <0.01 was considered statistically significant.

RESULTS

Adult Rat Kidney

Expression of EGF. In adult rat kidney, EGF immunoreactivity was observed in the cortex and outer medulla (Fig. 1). There was no immunostaining in the inner medulla, and the boundary between the outer and inner medulla was well defined. EGF immunostaining was predominantly observed in the outer and inner stripe of the outer medulla (Fig. 1, A and C) and was also found in some of the tubular profiles in the cortex (Fig. 1B).

Double immunohistochemistry. To identify each of the tubular profiles, we used different histochemical techniques: the antibody NKCC2 for TAL, nNOS for the MD, TSC for DCT, and CaBP for DCT2 and CNT. EGF immunoreactivity was observed in the NKCC2-positive TAL in the outer medulla and cortex (Fig. 2, A and A’), but there was no immunolabeling in the nNOS-positive MD (Fig. 2, B and 2B’). In the cortical labyrinth, EGF-labeling was prominent over region 1 of the TSC-positive DCT, but the labeling did not overlap region 2 of the CaBP-positive DCT or the CNT (Fig. 2, C, C’, and C’).

Electron microscopic immunocytochemistry. Electron microscopy showed the presence of EGF immunoreactivity on the apical plasma membrane and intracellular vesicles, including the Golgi complex of DCT (Fig. 3A). In contrast, the distribution of EGF immunolabeling in the medullary TAL was more diffuse than in the DCT cells, being found throughout the cytoplasm, with heavy labeling in perinuclear regions but not in the basolateral membrane (Fig. 3B). In cortical thick ascending limb, smooth-surfaced cells (SCC) have strong EGF immunoreactivity (Fig. 3C). In contrast, weak EGF immunoreactivity is seen only in a few vesicles of the rough-surfaced cell (RSC) with well-developed microvilli (Fig. 3D). In addition, the intensity of EGF immunolabeling of the TAL was stronger than in the DCT.

Developing Rat Kidney

Expression of EGF. No EGF-positive cells were found in fetal kidney. At E16, E18, and E20, EGF immunoreactivity was undetectable in any of the tubular segments (Fig. 4A). EGF-positive tubular profiles first appeared at P3 in the TAL, between the cortex and medullary regions in the middle region of the differentiating loops of Henle. No labeled profiles

![Fig. 3. Transmission electron micrographs illustrating EGF immunoreactivity in adult rat kidney. A: EGF immunoreactivity is present along the apical membrane and vesicles (arrows) in the apical portion of the cytoplasm of the DCT. Note the EGF-positive Golgi complex (G). Bar = 3 μm. B: intensity of EGF immunoreactivity of the medullary TAL (mTAL) cell is stronger than that of DCT cell in A. S3 is a segment of proximal tubule. Bar = 4 μm. C: in the cortical TAL (cTAL), smooth-surfaced cells (SSC) have EGF immunoreactivity. In contrast, weak EGF immunoreactivity is seen only in a few vesicles of the rough-surfaced cells (RSC) with well-developed microvilli (arrows). Bar = 5 μm.](http://ajprenal.physiology.org/)}
were found in the outer cortex or the inner medulla (Fig. 4B). At this age, EGF-negative cells could be detected among the EGF-positive cells (Fig. 4C). By P7, the pattern of EGF expression was very similar to that observed at P3, but the EGF-positive cells in the loop of Henle were more numerous and more intensely immunoreactive (Fig. 4D). By P14, EGF-positive cells could be found in the outer cortex and had begun appearing in the cortical labyrinth (Fig. 4E). By P21, the distribution of EGF had spread to the inner stripe of the outer medulla with a distribution similar to that seen in adult kidney (Fig. 4F).

**Double immunohistochemistry.** To determine whether EGF was expressed in the developing loop of Henle, serial sections were cut and double labeled. EGF-positive cells were first detected at P3 in the NKCC2-positive medullary TAL, the differentiating loop of Henle (Fig. 5, A and B). By P7, most of the TAL cells in the outer medulla and the inner cortex had become EGF immunoreactive, with more intense staining than that seen in the P3 kidney (Fig. 5D). In contrast, there was no staining in the DCT of the outer cortex at this age (figure not shown). By 14 days, EGF-positive cells were found in the initial portion of DCT, near the MD, but the staining was weak compared with that in adult kidney (Fig. 5E).

During renal development, the TAL contains both EGF-positive and EGF-negative cells that are clearly separable by light microscopy (Fig. 4C). We investigated double immunostaining for Na-K-ATPase and nNOS using serial sections cut from a postnatal kidney. Na-K-ATPase immunoreactivity was seen on the basolateral plasma membrane of the loop of Henle. Both EGF-positive and EGF-negative cells were well stained for Na-K-ATPase and were indistinguishable (Fig. 5F). EGF was not expressed in the macula densa during renal development (Fig. 4E).
Electron microscopic immunocytochemistry. EGF immunoreactivity was localized in the apical plasma membrane, the intracellular vesicles, and the Golgi complex in the stained semithin sections. EGF-positive vesicles were located in the supranuclear region and were attached to the apical plasma and the nuclear membrane (Fig. 6, A and B). EGF-positive cells in the TAL were smooth surfaced with few microvilli (Fig. 6B), whereas EGF-negative TAL cells had more numerous apical microvilli (Fig. 6C).

BrdU Staining (Proliferation Index)

Figure 4C shows that the immunostaining for EGF is heterogeneous in the TAL, with both positive and negative immunostained cells present. We measured the proliferation rates of EGF-expressing cells using BrdU staining at P3 and P7 to determine more precisely the differences between the positive and negative cell populations. These observations are relevant because the differentiating TAL of the medullary ray in the inner cortex and in the outer stripe of the outer medulla is a major site for cell proliferation during renal development (5).

The number of BrdU-positive nuclei, expressed as a percentage of the total number of EGF-positive cells at P3 and P7, are shown in Table 1. NKCC2 was used to identify the TAL (Fig. 7, A and B). Among the TAL cells, the largest number of BrdU-positive nuclei was observed in the TAL in the outer medulla at P3 (7.02%) and P7 (4.46%). In contrast, there were few BrdU-labeled EGF-positive cells in the medullary TAL (between 0.86 and 2.31%) (Fig. 7, C and D).

DISCUSSION

This study was designed to investigate more thoroughly the distribution of the EGF and whether it coincides with proliferation along the loop of Henle in developing rat kidneys (Fig. 8). The main result of the study was demonstration of EGF immunoreactivity in the adult rat kidney, which was localized to the TAL and DCT1. These results differ from previous studies using rat (25, 32), mouse (31, 33), and rabbit kidneys (34). Previous studies have examined the immunoreactivity of EGF in the different segments without demonstrating a difference between DCT1 and DCT2 (25, 31, 32–34). Dorup (9) reported ultrastructural distinctions between cells in the early and later part of the DCT in rat kidney. Loffing (19) proposed the subdivision of the DCT into DCT1 (sodium/calcium exchanger negative) and DCT2 (sodium/calcium exchanger positive). DCT2 cells, which occupy a transitional region between the DCT and the CNT, display features of both DCT1 and CNT cells. In particular, DCT2 contains TSC, characteristic of the DCT (19), but also expresses CaBK (20, 27), which is characteristic of the CNT. In addition, intercalated cells are present between DCT2 cells (9, 21). In our study, EGF immunoreactivity was found only in the DCT1 region and not in the DCT2 region, which expressed both TSC and CaBP. This study confirmed that EGF is produced and secreted from both the TAL and DCT1 in adult rat kidney.

In developing kidney, EGF was first detected at P3 in the differentiating TAL of the medullary ray of the outer medulla; it had gradually spread to the inner cortex by P7. At P14, EGF first appeared in the distal tubule except for the macula densa. Finally, at P21, EGF immunoreactivity was found in the TAL,

Fig. 5. Micrographs of serial sections illustrating NKCC2 (A and C) and EGF (B and D) in the OM from P3 (A and B) and P7 pups (C and D) and double immunolabeling for EGF (brown) and nNOS (blue; E) or Na-K-ATPase (blue; F) in the Co at P14. A and B: apical staining of NKCC2 in the TAL (*) coincides with weak EGF staining (arrows) at P3. C and D: in a P7 pup, strong EGF labeling overlaps with NKCC2-positive TAL (C, D). E and F: EGF immunoreactivity is not expressed in the macula densa (MD; arrow), whereas it is present in D1 in a P14 animal. Magnification: ×450 (A–D); ×480 (E and F).
extending throughout the inner stripe of the outer medulla to the outer cortex and the DCT of the cortical labyrinth.

EGF promotes cell proliferation from numerous cell culture studies using kidney cells and those of other organs (1, 13, 24), but the localization of EGF in renal development in vivo has been studied little. Moreover, there is controversy concerning the localization and expression time of EGF during development. Several studies in mice have stated that EGF appeared 3–5 days after birth (33); Gattone et al. (12) reported the presence of EGF after day 6 and Popliker et al. (28) after 2 wk. Our study in rats shows that EGF-positive cells can be detected at P3 and are localized in the differentiating TAL of the medullary ray. In developing rat kidney, the ureteric bud and metanephrogenic blastema converge and begin the full development of a new nephron in the nephrogenic zone from E13 to P7. In this study, we found that between P3 and P7, EGF-positive cells appear in the TAL of the corticomedullary junction, one of the main sites of proliferation during renal development. However, developing EGF-negative cells were detected among the EGF-positive cells. Nouwen et al. (25) reported that there were EGF-negative cells among positive ones in the TAL epithelium of the outer stripe in a light microscopic study. Our results, however, demonstrate that EGF-negative cells are found both in the TAL of the outer stripe of the outer medulla and in the cortex. We used three different methods to identify the features of EGF-positive and -negative cells. To confirm the presence of immature EGF-negative cells in the developing kidney, we used double staining for EGF and Na-K-ATPase α1, which exists in the basolateral membrane of the loop of Henle. No difference was found in sodium transport proteins staining between the EGF-positive and -negative cells during renal development. We have previously reported the cell proliferation rate in the developing process of the loop of Henle and the TAL of the outer medulla and have established the importance of that region for cell proliferation during renal development (5). In this study, we did not find a significant difference in the cell proliferation rate of EGF-positive cells in the TAL; in fact, cell proliferation was lower in the EGF-stained cells (Table 1).

However, electron microscopy confirmed that EGF-positive cells were smooth-surfaced with less well-developed microvilli, whereas EGF-negative cells were rough, with better developed microvilli. Allen and Tisher (2), using scanning electron microscopy, reported the presence of two types of epithelial cells in the thick ascending tubules that were distinguishable by the degree of development of the surface microvilli. Yoshitomi et al. (36) and Tsuruoka et al. (35) reported that these two types of cells have different electrophysiological characteristics; more recently, Nielsen et al. (23) reported that BSC1 immunoreactivity differs between the rough- and smooth-surfaced cells. However, we did not find a difference in the immunoreactivity staining of sodium transport proteins but observed that the proliferation rate was lower for positive cells than for negative cells. Further research is required to explain the functional significance of these observations.

At P14, EGF immunoreactivity appeared in the cortical TAL and the DCT of the cortical labyrinth. We also found that after P21, immunoreactivity dramatically increased to levels similar to those seen in adult animals. It is suggested that EGF in the kidney has a role in differentiation and growth rather than in cell proliferation, as EGF expression is found in kidneys immediately after birth and expression is increased at P7, the

Table 1. Percentage of BrdU-labeled cells in the NKCC2/EGF-positive TAL of loop of Henle in the postnatal rat kidney

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<td>NKCC2-positive TAL cells, %</td>
<td>7.02 ± 3.14</td>
<td>4.46 ± 1.18</td>
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<tr>
<td>EGF-positive cells, %</td>
<td>2.31 ± 1.06*</td>
<td>0.86 ± 0.77*</td>
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Values are means ± SD expressed as a percentage of Na-K-2Cl cotransporter (NKCC2)/epidermal growth factor (EGF)-positive thick ascending limb (TAL) cells in outer medulla. BrdU, 5'-brom-2'-deoxyuridine; P3 and P7, postnatal 3- and 7-day-old rats, respectively. *P < 0.01
time when the nephron ends formation and differentiation and maturation begin. The EGF receptor is present in the kidney before birth and is lost gradually after birth. However, as stated above, EGF in the developing kidney appears at P3, and EGF produced in the kidney has no effect upon the incipient process of kidney development. It is believed that EGF and transforming growth factor-α participate in the development of the kidney by affecting EGF receptors at birth.

Apoptosis plays an important role in rat kidney morphogenesis, being mainly present in the nephrogenic zone before birth and in the renal papilla after birth. After birth, apoptosis in the renal papilla participates in the elimination of thick ascending epithelial cells and in the formation of the ascending thin limb epithelium (17). It has been reported that apoptosis is involved in the temporary appearance and subsequent removal of a large number of intercalated cells at early stages of development. Coles et al. (7), having observed the decrease in apoptosis following injection of EGF, suggested that EGF might control apoptosis. Our study suggests the possibility that EGF influences the control of apoptosis as EGF appears at P3, with EGF expression localized to the inner cortex and outer medulla, a region where apoptosis is rarely present. We also observed a
sudden increase in EGF expression at P14, approximately at the time when apoptosis ceases. These results suggest that EGF expression in kidney development may be involved not so much in cell proliferation as in the differentiation and maturation of the distal tubule.

In summary, our study has demonstrated that EGF immunoreactivity is restricted to the TAL and DCT1 of adult rat kidney and that there are two cell types in the developing TAL: those that are EGF positive with smooth surfaces and those that are EGF negative with rough surfaces. In addition, EGF expression does not coincide with cell proliferation in the developing loop of Henle.

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