FULL PAPER *Anatomy*

Compensatory Renal Growth in Uninephrectomized Immature Rats: Proliferative Activity and Epidermal Growth Factor

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ABSTRACT. Compensatory response to uninephrectomy in immature animals is stronger compared with that in adult ones and the response is due mainly to renal cell proliferation. The present study explored to show the growth pattern of the remaining kidney immediately after uninephrectomy in immature rats with special reference to proliferating activity and epidermal growth factor (EGF). Immunolocalizations of proliferating cell nuclear antigen (PCNA) and EGF in immature rat kidney were examined during the first three days after uninephrectomy. Semi-quantitative analysis of the expression of preproEGF mRNA was performed. One day after the operation, the PCNA positive cell ratios in the glomeruli and the proximal tubules were significantly higher in unilaterally nephrectomized (UNx) rats than in sham-operated (Sham) rats. In UNx and Sham rats, the proximal and distal tubular cells showed positive reactions to EGF antibody. The positive reaction of proximal tubules to EGF antibody was weaker in UNx than in Sham rats 1 day after the operation, while the degree of reactivity was not different between UNx and Sham rats 3 days after the operation. The level of expression of preproEGF mRNA in the kidney was significantly lower in UNx than in Sham rats 1 day after the operation. These results indicate that unilateral nephrectomy in immature rats causes increased proliferative activity and decreased expression of EGF in the remaining kidney during the early period of compensatory renal growth.

KEY WORDS: EGF, glomerulus, PCNA, proximal tubules, uninephrectomy.

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Compensatory renal growth after unilateral nephrectomy has been intensely investigated [5, 12, 18] and includes cellular hypertrophy, hyperplasia, and apoptosis [30]. In immature animals, the compensatory response to uninephrectomy is stronger than in adult ones [6]. The response is largely due to hyperplasia in immature animals, and due to hypertrophy in adult animals [8]. On the other hand, a variety of polypeptide growth factors regulate cell proliferation during development [1]. Of these growth factors, epidermal growth factor (EGF) was first isolated from mouse submandibular glands [4]. The kidney is the organ next to the salivary glands in secreting a large amount of EGF [11]. EGF is involved in the process of preventing renal tubular scarring in 5/6 nephrectomized immature rats by protein restriction [17]. Addition of EGF to renal organ culture medium causes an increased uptake of bromodeoxyuridine (BrdU) [26]. Absence of EGF in the conditions after acute renal failure, where its mitogenic properties would be most appropriate, suggests that the EGF of renal origin is not acting as a mitogen during kidney regeneration [20]. Recently, we have reported that EGF plays an important role in both cell proliferation and maturation of the proximal tubules in perinatal rat kidney [23]. These findings lead us to expect that some changes in proliferative activity and localization of the growth factor would be occurred in the remaining kidney of immature rats immediately after uninephrectomy.

Therefore, the present study was designed to clarify the changes in proliferative activity and the expression of EGF in compensatory renal growth immediately after uninephrectomy in immature rats.

MATERIALS AND METHODS

Animals and tissue processing: Wistar rats were maintained under the conditions of regulated room temperature (24 ± 1°C), humidity (55 ± 5%), and lighting (from 06:00 to 20:00). The animals were given a commercial diet (NMF, Oriental Yeast Co., Tokyo, Japan) and water both *ad libitum*. The present study was performed in accordance with the Guidelines for Animal Experimentation of Graduate School of Life and Environmental Sciences, Osaka Prefecture University, Japan. Three weeks old rats (30 to 40 g in body weight) were purchased from CLEA Japan Inc. (Suita, Osaka, Japan) and divided into two groups: uninephrectomized (UNx, 20 animals) and sham-operated (Sham 20 animals) rats. The operations were performed under ether anesthesia according to the method described in our previous report [24] as follows. Under ether anesthesia, animals were subjected to left dorsolateral laparotomy and the left kidney was pulled out to expose the renal vessels and ureter. In UNx rats, the vessels and ureter were ligated with cotton thread and cut between the kidney hilus and ligated portion

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to remove the kidney. In Sham rats, the thread were passed around the vessels and ureter and removed. The ligated portion or kidney was returned to abdominal cavity and the incision was sutured. After surgery, antibiotics and analgesics were administered. On the 1st, 2nd, or 3rd postoperative day, the rats were decapitated under ether anesthesia. The right kidney was quickly removed and divided into some blocks. The blocks were fixed in methanol-Carnoy solution [a mixture of methanol, chloroform, acetic acid (6:3:1)] for 24 hr, dehydrated through a graded series of alcohol, and embedded in Tissue Prep (Fisher Scientific, Fair Lawn, NJ, U.S.A.). Sections prepared at 6 μm were used for immunohistochemical examination for the localization of PCNA and EGF. The renal sections obtained from adult rats were also examined for the localization of EGF.

Immunohistochemical procedures: After deparaffinization with xylene, the sections were transferred to distilled water through a degraded series of ethanol and rinsed in phosphate buffered saline. The sections were incubated with mouse anti-human PCNA antibody (19A2, Coulter Immunology, Hialeah, FL, U.S.A., 1:160) at 4°C overnight. Then, the sections were incubated with biotinylated rabbit anti-mouse immunoglobulins antibody (BioGenex Laboratories, San Roman, CA, U.S.A., 1:50) and streptavidin conjugated peroxidase (Zymed Laboratories, South San Francisco, CA, U.S.A., 1:50) for 30 min, respectively. The EGF immunostaining was performed as follows: the sections were incubated with rabbit anti-rat EGF antibody (IGG, Nashville, TN, U.S.A., 1:1,200) at 4°C, overnight. Then, the sections were incubated with biotinylated goat anti-rabbit IgG antibody (1:200) and an avidin-biotin-peroxidase complex (1:200) for 30 min respectively. Finally, the sections were incubated with diaminobenzidine for 2 to 5 min. To identify the proximal tubules, the periodic acid Schiff (PAS) reaction was conducted on the PCNA immunostained sections and on the sections close to the EGF immunostained sections. Identification of renal tubule morphology was performed by conventional staining methods. Negative controls were produced by omitting the primary antibody during the immunohistochemical procedure. No positive immunoreactivity was recognized when anti-rat EGF antibody was preincubated with an excess of antigen (10 μg/m*l* rat EGF, IGG, Nashville, TN, U.S.A.).

Semi-quantitative RT-PCR of preproEGF mRNA in rat kidney 1 day after nephrectomy: Total RNA was extracted from the kidneys of UNx and Sham rats using ISOGEN RNA isolation protocol (NIPPON GENE, Toyama, Japan). The RNA was resuspended in 50 μ*l* diethyl-pyrocarbonate-treated water. Complimentary DNA was prepared from 3 μg of each RNA using Ready-To-Go^{TM} T-Primed First-Strand Kit (Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.). PCR was performed using 2 μ*l* of the subsequent cDNA preparations. cDNA was added to reaction mixture containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.001% (W/V) gelatin, 0.2 mM dNTP, 0.5 mM of both 5' and 3' primers, and 2.5 U Taq polymerase (AmpliTaq Gold, Perkin Elmer, Hayward, CA, U.S.A.) in total volume of 50 μ*l*. RT-PCR amplification of β-actin mRNA was carried out with the same sample as a positive control. For preproEGF mRNA, PCR conditions were 24, 27, and 30 cycles with denaturation at 94°C for 1 min, annealing at 57°C for 1 min, and extension at 72°C for 1 min, with a hot start at 95°C for 12 min and final extension at 72°C for 10 min. RT-PCR products were separated on 1.5% agarose gels, stained with ethidium bromide and visualized under ultra violet light. The primer sequences were designed from cDNA sequence for rat preproEGF mRNA [27] and β-actin [21] in previous papers. The actual sequences of primers were as follows: sense primer 5'-GGTCCGAAACAGTA ACACAG-3', antisense primer 5'-CTTCTGTCTCCAG-GAAGTCA-3' for preproEGF, sense primer 5'-AACG-GTCTCACGTCAGTGTA-3', antisense primer 5'-GTATCCACGGCATAGATGGT-3' for β-actin. The primers correspond to sequence in 5' and 3' region resulting in amplification of a splice of a size of 528 base pairs (preproEGF) and a size of 332 base pairs (β-actin). For β-actin mRNA, the same PCR condition as for preproEGF mRNA was used except for the cycles (21, 24 and 27) and the annealing temperature (59°C). Semi-quantification was achieved during the exponential phase with 24 cycles for β-actin and 27 cycles for preproEGF. Integrated density of the expression was determined with NIH Image software. The relative expression level of preproEGF mRNA was calculated as a percentage relative to the value of the β-actin expression.

Determination of PCNA positive cell ratio: To determine the PCNA positive ratio in the glomerulus, 10 glomeruli were used. To determine the ratio in the proximal tubules, more than 500 nuclei were used in the proximal convoluted and straight tubules, respectively. The nuclei positive and negative to PCNA were counted and the ratio of positive nuclei to total nuclei was expressed as a percentage.

Statistics: The data were expressed means ± SEM. The differences between UNx and Sham rats were analyzed with the Student's *t* test. A *P* value less than 0.05 were identified as statistically significant.

RESULTS

Changes in renal weight: After the operation, the renal weight is higher in UNx than in Sham rats and significant increase in renal weight by uninephrectomy was noted on 1 and 3 postoperative days (Table 1).

Immunolocalization of PCNA and PCNA positive cell ratio: PCNA positive cells were observed mainly in glomeruli and proximal tubules and observed in distal tubules little in both UNx and Sham rats. The positive reaction in the glomerulus is observed in endothelial and mesangial cells. PCNA positive cells were more frequently observed in UNx rats than in Sham rats (Fig. 1). PCNA positive cell ratios of the glomerulus and proximal convoluted and straight tubules were significantly higher in UNx than in Sham rats on each postoperative day (Table 2).

Immunolocalization of EGF: In the kidney of adult rats,

Table 1. Changes in renal weight in uninephrectomized (UNx) and sham-operated (Sham) rats

		Day after operation		
		1	2	3
		Mean ± SEM	Mean ± SEM	Mean ± SEM
Sham	(5)[a)]	195.7 ± 5.5	201.3 ± 10.0	222.2 ± 8.0
UNx	(5)	230.3 ± 6.8*	236.1 ± 8.9	271.8 ± 10.7*

a) No. of animals appear in parentheses.
*, Significantly different from age-matched Sham rat (P<0.05).

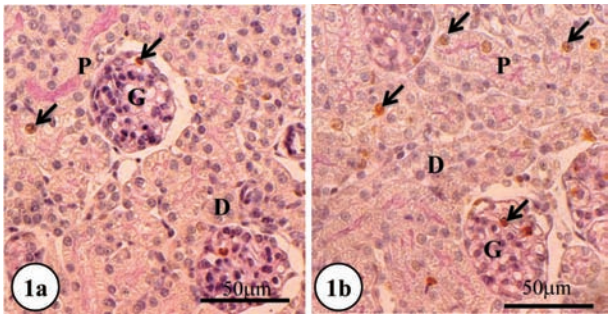

Fig. 1. Kidney sections stained with PCNA antibody and PAS. a: A Sham rat 1 day after operation. The PCNA positive cells (arrows) are rarely seen. b: An UNx rat 1 day after the operation. The PCNA positive cells (arrows) are seen in the glomeruli and proximal tubules. The PCNA positive cells are greater in number when compared with the section of an age-matched Sham rat in Fig. 1a. Counter stained with hematoxylin. G, glomerulus; D, distal tubule; P, proximal tubule.

EGF positive cells were observed in the distal tubules but not in the proximal tubules (Fig. 2a). The positive reaction was not observed in the section, which was stained with the antibody preincubated with an excess antigen (Fig. 2b). In UNx and Sham rats, EGF positive cells were localized in the proximal tubules and the distal tubules, while the positive cells were not found in the glomeruli. One day after the operation, the proximal tubular cells showed weaker reaction to EGF antibody in UNx rats than in Sham rats (Fig. 2c and 2d), while no difference in the reaction to EGF antibody was noted (Fig. 2e and 2f). The degree of reactivity to EGF was the same in both groups 3 days after the operation (Fig. 2g and 2h).

Semi-quantitative RT-PCR of preproEGF mRNA in the kidney 1day after nephrectomy: The level of expression of preproEGF mRNA was significantly lower in UNx than in Sham rat (Fig. 3).

DISCUSSION

In the adult kidney, differentiated nephrons are relatively quiescent, with few cells undergoing mitosis [8]. Compensatory response after renal mass ablation shows increase both in cell size and cellular protein content without increment in cell number [8]. The renal growth largely occurs by hypertrophy rather than hyperplasia of the remaining nephrons [2]. In contrast, in immature rats, the increase in kidney mass immediately after nephrectomy is mainly due to hyperplasia, whereas 2 weeks after nephrectomy both hyperplasia and hypertrophy processes equally participate [15].

Since the PCNA positive cells in distal tubules were little

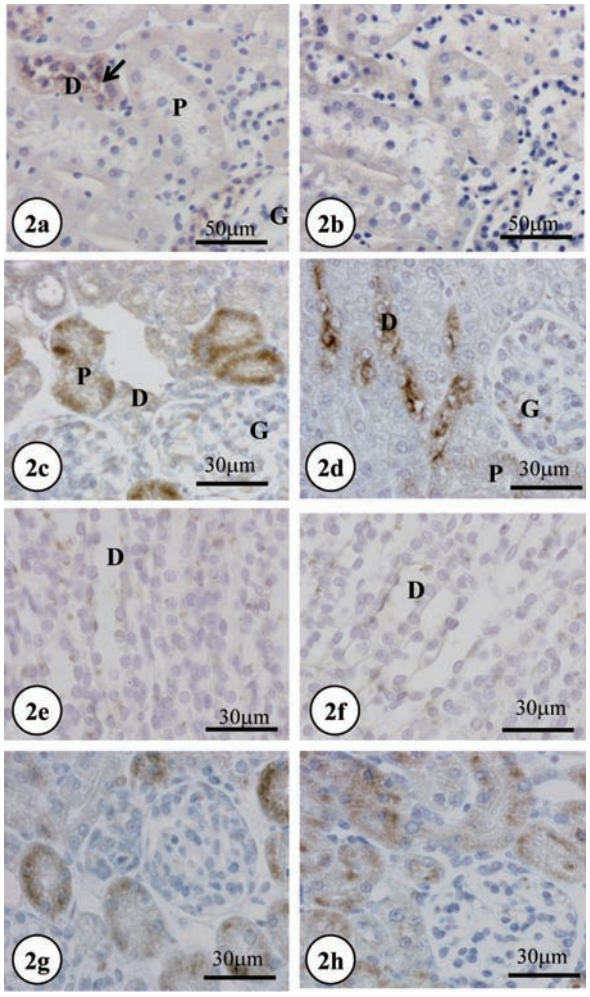

Fig. 2. Kidney sections stained with EGF antibody. a: Adult rat kidney. EGF positive reactions (arrows) are seen in the distal tubules. b: Absorptive test of EGF in adult rat kidney. No positive reaction is seen. c: A Sham rat 1 day after operation. EGF positive cells are seen in the distal tubules and proximal tubules. d: An UNx rat 1 day after operation. Positive reaction of the proximal tubules to EGF is weak when compared with the section of an age-matched Sham rat in Fig. 2c. e: Sham rat 1 day after operation (medullary region). EGF positive cells are seen in the distal tubules. f: An UNx rat 1 day after operation (medullary region). Positive reaction of the distal tubules to EGF is similar to the section of an age-matched Sham rat in Fig. 2e. g: A Sham rat 3 day after operation. EGF positive cells are seen in the distal tubules and proximal tubules. h: An UNx rat 3 days after operation. Positive reaction of the proximal tubules to EGF is similar to the section of an age-matched Sham rat in Fig. 2g. Counter stained with hematoxylin. G, glomerulus; D, distal tubule; P, proximal tubule.

Table 2. PCNA positive cell ratio (%) of glomerulus and proximal tubules in uninephrectomized (UNx) and sham-operated (Sham) rats

	Day after operation		
	1	2	3
	Mean ± SEM	Mean ± SEM	Mean ± SEM
Glomerulus			
Sham (5)[a)]	1.99 ± 0.34	2.40 ± 0.57	2.27 ± 0.24
UNx (5)	7.12 ± 1.54*	6.24 ± 1.18*	6.21 ± 0.52*
Proximal convoluted tubules			
Sham (5)	0.77 ± 0.28	1.34 ± 0.27	1.43 ± 0.38
UNx (5)	5.03 ± 0.26*	4.74 ± 0.76*	3.34 ± 0.63*
Proximal straight tubules			
Sham (5)	5.04 ± 0.68	5.87 ± 0.83	5.41 ± 0.48
UNx (5)	12.11 ± 0.94*	13.27 ± 1.10*	12.09 ± 0.72*

a) No. of animals appear in parentheses.
*, Significantly different from age-matched Sham rat ($P<0.05$).

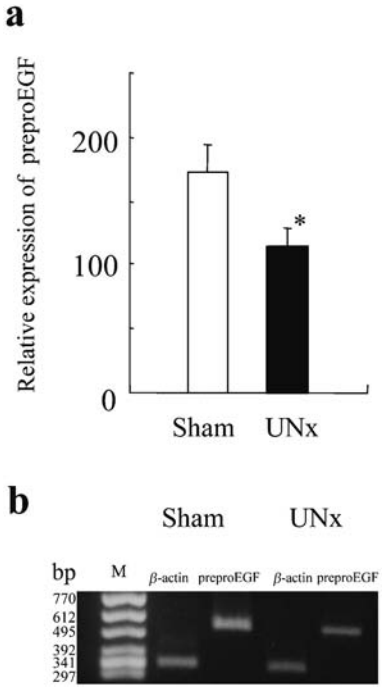

Fig. 3. Semi-quantitative RT-PCR analysis of the expression of preproEGF mRNA in the kidney 1 day after uninephrectomy. a: The relative expression level of preproEGF was calculated as a percentage relative to the value of the β-actin expression. Data are expressed as the means ± SEM of 3 or 4 individual experiments. UNx, UNx rats. Sham, Sham rats. *, Significantly different from sham rats ($P<0.05$). b: Representative separation by 1.5% agarose gel electrophoresis of the RT-PCR products amplified from total RNA extracted from the kidney of UNx and Sham rats. DNA marker (M) is shown in the left lane. The expected product sizes were 332 bp for β-actin and 528 bp for preproEGF.

observed in the present study and it has been reported that the removal contralateral kidney induces an evident increase in cell proliferation, especially in proximal tubules in young uninephrectomized rats [8], the present study focused on changes in the proliferative activity of glomeruli and proximal tubules in remaining kidney after nephrectomy. The present result that the PCNA positive cell ratios in the proximal convoluted and straight tubules were significantly higher in UNx than in Sham rats on each postoperative day indicates that an increase in proliferative activity in the proximal tubules during the early stage of compensatory renal growth in immature rats as shown in Table 1. This is consistent with the report by Kanda *et al.* [14] on the remaining kidney stained with anti-BrdU antibody where the proliferation of cortical tubular cells started 24 hr after uninephrectomy of adult mouse kidney. Further, the present finding that the PCNA positive cell ratio in glomerulus was significantly higher in UNx than in Sham rats on each postoperative day showed the increase in proliferative activity in glomerulus after uninephrectomy. With regard to human fetal kidney immunostained with the PCNA antibody, Nagata *et al.* [19] reported that cell proliferation is not found in glomerular epithelium in mature type of glomerulus. Pavenständt *et al.* [25] reported that the proliferation arrest of podocytes was seen in mature glomerulus. In the present study, PCNA positive cells in glomerulus were seen in endothelial and mesangial cells, but not in epithelial cells. Further, EGF positive cells were not observed in the glomerulus and EGF receptor positive reactions are not seen in mature type of glomerulus [22]. Based upon these findings, it is suggested that EGF is not involved in the cell proliferation of glomerulus but rather in that of the proximal tubules.

Toubeau *et al.* [28] and Jung *et al.* [13] have observed immunolocalizations of EGF in distal convoluted tubules and thick ascending limb of Henle's loop of adult rat kidney. In the present study, EGF positive cells were observed not in the proximal tubules but in the distal tubules in adult kidney. Since the positive reaction was vanished by the preincubation of antibody with an excess of antigen, the specificity of

the antibody was confirmed. However, EGF positive cells were observed in proximal tubules as well as in distal tubules of both UNx and Sham rats (3 weeks old rats). This suggests that the 3 weeks old rat kidney is immature and shows different localization of EGF from adult rat kidney. This notion is supported by the report of Raaberg *et al.* [29] on the rat kidney that immunohistochemically positive EGF is observed in the proximal tubules at birth and that the degree of positive reaction decreases slowly with age.

There are different accounts of the role of EGF in renal tissues as follows. Addition of EGF to renal organ culture medium increases uptake of BrdU [26]. Cell proliferation occurs immediately after the decrease of renal EGF and its receptor (EGFR) in the rat experimentally subjected to acute renal failure [28]. In the present study, 1 day after uninephrectomy the reactivity of the proximal tubules to EGF antibody was weak in UNx rats, and 3 days after the operation no difference in the reactivity was seen between the UNx and Sham rats. And 1 day after uninephrectomy, the reaction to EGF antibody in medullar region of the kidney was not change between UNx and Sham rats. Further, the expression of preproEGF mRNA was significantly lower 1 day after the uninephrectomy when compared to that of control rats. These findings indicate that EGF has an inhibitory effect on the proliferation of normally developing cells and that a decrease in the expression of EGF causes elevated proliferative activity in the proximal tubules of uninephrectomized rats. This idea is supported by several lines of evidences. In the unilaterally ureter obstructed rat model, an administration of EGF causes a decrease of proliferative activity in the contralateral kidney [3]. In the developing kidney, EGF expression appears as nephrons mature [20] and exogenous EGF delays the development of loop of Henle by reducing both apoptosis and cell proliferation [16]. Robust EGF synthesis is clearly a characteristic of the mature kidney rather than that of a rapidly growing fetal organ [9]. Renal EGF is undetectable in human fetus [10] and renal EGF content is increased from days 6 to 21 after birth in mice [7].

Consequently, the present study indicates that uninephrectomy in immature rats causes an elevation of proliferative activity and a decreased expression of EGF in the remaining kidney during the early stage of compensatory renal growth.

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