Transient increase in renal epidermal growth factor content after unilateral nephrectomy in the mouse

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Abstract. A sensitive enzyme-linked immunosorbent assay for mouse epidermal growth factor was established for measurement of the content of epidermal growth factor in the remaining kidney after uninephrectomy. In 5-week-old male mice, the renal epidermal growth factor content before uninephrectomy was 355±97 ng/g wet tissue with a 2.1-fold increase on the first day after uninephrectomy, whereafter it gradually decreased. In 15-week-old mature male mice, the renal epidermal growth factor content increased 1.7-fold on the first day after uninephrectomy. Immunohistochemical analysis showed that epidermal growth factor was present in the distal tubular cells and that the staining intensity was increased on the first day after uninephrectomy. During the course of compensatory renal growth, no significant alteration of epidermal growth factor content was observed in plasma or in the submaxillary gland. Our data suggest that the increase in renal epidermal growth factor content after uninephrectomy is due to an increased production of epidermal growth factor in the kidney itself. The significance of this phenomenon is discussed.

Compensatory renal growth after unilateral nephrectomy is a well-known phenomenon and it includes cellular hyperplasia as well as hypertrophy. Previous studies showed that the hyperplasia was transient and occurred early after uninephrectomy, whereas the hypertrophy was constant (1). Furthermore, the compensatory renal hyperplasia is more dominant in young than in mature animals (2,3). A possible role of some endocrine factors in the compensatory renal growth has also been reported (4-8). Thus, insulin-like growth factor-I increases in the kidney after uninephrectomy and acts as a paracrine factor (9,10).

Epidermal growth factor (EGF), which is abundant in kidney, is a potent growth stimulator for renal tubular cells (11-13), and it also regulates some renal functions (14,15). However, the physiological significance of renal EGF on compensatory renal growth is still not clearly known. We have measured the renal immunoreactive EGF after unilateral nephrectomy using an enzyme-linked immunosorbent assay (ELISA).

Material and Methods

EGF was purified by the method of Savage & Cohen (16). Human recombinant insulin-like growth factor and bovine basic fibroblast growth factor (bFGF) were obtained from TOYOBO, Osaka, Japan. Human EGF was purchased from Boehringer Mannheim, FRG. Rat transforming growth factor-α (TGF-α) was from Bio Tope, Redmond, WA. Human platelet-derived growth factor (PDGF) was from R&D systems, Minneapolis, MN. Pepsin, Freund’s complete and incomplete adjuvant were from Gibco, Grand Island, NY. Microwell plates were from Nunc, Roskilde, Denmark. Protein A Sepharose was from Pharmacia, Uppsala, Sweden. Biotin-N-hydroxy-succinimide ester, peroxidase conjugated streptavidin, and o-phenilene diamine were from Zymed Laboratories, San Francisco, CA. Bovine serum albumin was purchased from Sigma, St. Louis, MO. Male ddY mice were obtained from Otsuho Experimental Animals, Nagasaki, Japan.
Preparation of antibody against mouse EGF
A male 10-week-old New Zealand white rabbit was immunized intradermally with purified EGF with Freund's adjuvant every 2 weeks and bled after the fifth immunization. IgG fraction was purified using Protein A Sepharose affinity chromatography and purified IgG was fragmented to Fab' as described (17). A part of this Fab' fragment was further biotinylated (18).

Preparation of plasma or homogenate of mouse submaxillary glands and kidney
Each set of three male ddY mice were anesthetized lightly with ether and a left unilateral nephrectomy or a sham operation was performed through a left flank incision. On pre-established days after uninephrectomy or sham operation, the blood was collected from the anesthetized mice with EDTA (final concentration: 0.008 mol/l) by cardiac puncture. Blood samples were centrifuged to remove the blood cells. Then the plasma was treated with acid-ethanol (19), centrifuged to remove denatured protein, and the supernatant was lyophilized and stored at -20°C until assay.

Next, kidney and submaxillary glands were removed from the bled mice, homogenized in 1 mol/l acetic acid at 0-4°C, and centrifuged at 5000 x g. The supernatants were lyophilized and stored at -20°C.

These samples were resolved in Buffer A and used for ELISA immediately after reconditioning.

Assay for measurement of immunoreactive EGF concentration with the ELISA system
Nunc Microwell plates were coated with anti-mouse EGF Fab' fragment in coating buffer (0.05 mol/l carbonate buffer, pH 10.0, containing 0.15 mol/l NaCl). After this coating, the plates were washed with Buffer A (0.01 mol/l sodium phosphate buffer, pH 6.0, containing 0.15 mol/l NaCl, 0.001 mol/l MgCl₂, 0.5% BSA, and 0.05% Tween 20) and blocked with 0.1% BSA in Buffer A; then samples or standard EGF were added to the wells. After 60 min incubation of the plates at 37°C, wells were washed with Buffer A and biotinylated anti-EGF Fab' fragment was added to the wells and the plates were incubated for 60 min at 37°C. Then the wells were washed, peroxidase conjugated streptavidin was added, and the wells were incubated for 60 min. Finally, the wells were washed with Buffer A and o-phenylene diamine was added to the wells; the peroxidase reaction was stopped with 2 mol/l H₂SO₄. The absorbance was measured at 492 nm. Comparisons between mean values were conducted using Student's t-test.

Immunoblot analysis
Purified mouse EGF, homogenate of submaxillary gland and kidney were electrophoresed on SDS-polyacrylamide gels (16%) and transferred electrophoretically to a nitrocellulose membrane. The nitrocellulose membrane was blocked with 2% BSA solution and incubated with anti-mouse EGF Fab' fragments. The proteins were visualized by successive incubations with peroxidase-conjugated anti-rabbit IgG and 4-chloro-1-naphtol substrate.

Immunohistochemical staining of EGF
First, the formalin-fixed mouse renal tissues on the gelatin-coated slide glass were incubated with 0.3% H₂O₂ in absolute methanol for 30 min and then washed with buffer B (phosphate buffered saline, pH 7.2, containing 0.1% Tween 20 and 0.1% bovine serum albumin), whereafter it was again incubated with 1% bovine serum albumin for 20 min at room temperature. Next, the bovine serum albumin was removed and the tissues incubated with anti-EGF rabbit IgG (50 mg/l) or nonimmunized rabbit IgG for 60 min at 37°C. Following this, the tissues were washed with buffer B and incubated with biotinylated antirabbit IgG (10 mg/l) for 60 min at 37°C, washed with buffer B and incubated in a 200-fold diluted streptavidin-peroxidase complex for 30 min at room temperature. Finally, the tissues were washed with buffer B and the peroxidase reaction was performed in a dianisobenzidine solution containing 0.025% CoCl₂ and 0.02% NiSO₄.

Results

Enzyme-linked immunosorbent assay characteristics
Fig. 1 shows the immunoblot analysis using anti-

![Image](https://example.com/image1)

Fig. 1.
Immunoblot analysis of purified mouse epidermal growth factor (EGF) and homogenates of mouse submaxillary gland and kidney. One microgram of mouse EGF (lane A), 1 μg of submaxillary gland homogenate (lane B), and 10 μg of kidney homogenate were electrophoresed on SDS-polyacrylamide gel, transferred to a nitrocellulose membrane, and incubated with anti-mouse EGF IgG Fab' fragment.
mouse EGF Fab' fragment. Purified EGF expressed the single band, the molecular weight of which was about 6 kD. The homogenate of submaxillary gland or kidney also showed the same band as purified EGF. This result indicated that anti-mouse EGF antibody derived from our laboratory reacted only with mature 6 kD EGF.

Fig. 2 shows the typical dose-response curve of the ELISA system. This curve was almost linear between 0.025 and 1 ng of EGF per well. An analysis showed that in this assay system, the rate of cross-reactivity of rat TGF-α, human EGF, human IGF-I, and human PDGF was lower than 0.25% and that of bovine basic FGF was lower than 0.5%.

Fig. 3 shows the dilution curves for samples from control mouse serum and kidney. The slopes of serially diluted samples with buffer A are parallel to the standard EGF.

Thus, this ELISA system was specific for mouse EGF.

**Immunoreactive EGF content in plasma, kidney, and submaxillary gland from nephrectomized mice**

First, the renal immunoreactive EGF content was measured by the ELISA system. Fig. 4 shows the alteration of kidney weight as well as renal immunoreactive EGF content after unilateral nephrectomy. It was remarkable that renal EGF content increased transiently on the first day after unilateral nephrectomy (p<0.05 between control value and value on day 1) and decreased after the third day in both 5-week-old and 15-week-old mice. The rate of increase in renal EGF content was higher in 5-week-old mice (2.1-fold) than in 15-week-old mice (1.7-fold). The weight gain of the remaining
kidney was also greater in 5-week-old than in 15-week-old mice. The right renal EGF content on the first day after sham operation in three young mice (5-week-old) was 351±34 ng/g tissue, which was almost similar to normal control renal EGF concentration. These results indicated that the increase in renal immunoreactive EGF content after uninephrectomy was strongly related to the compensatory renal growth.

The submaxillary gland is a rich source of EGF in mice, and to clarify its role in the production of EGF after uninephrectomy, we measured submaxillary gland as well as plasma immunoreactive EGF concentration after unilateral nephrectomy. However, no significant alteration of the EGF concentration was observed in the submaxillary gland or in plasma during the course of compensatory renal growth (Fig. 5A and B).

**Immunohistochemical staining of EGF in renal tissue**
Immunohistochemical staining of EGF was also performed in both uninephrectomized and control mice in order to clarify the specific site and time of EGF production in the kidney, which is shown in Fig. 6A and B. Results showed that there was low EGF immunoreactivity in the distal tubular cells in normal kidneys. The intensity of the immunoreactivity, however, markedly increased after uninephrectomy (on day 1) which is consistent with the renal/EGF concentrations observed by the ELISA.

**Discussion**
The kidney itself produces EGF (20,21) and EGF is a potent growth stimulator for renal tubular cells (11-13). However, it is not clear whether or not EGF affects the compensatory renal growth after uninephrectomy. We therefore measured renal
EGF concentration during the course of compensatory renal growth and found that the renal immunoreactive EGF content increased on the first day after uninephrectomy and decreased gradually during the following days (Fig. 4). No alteration of plasma EGF concentration or submaxillary gland EGF content was observed during the compensatory renal growth. Salido et al. reported that renal EGF increased at the seventh day after uninephrectomy (22), and Oka et al. did not find any change of renal EGF content after uninephrectomy (23). However, none of them had measured the renal EGF content at the first day after uninephrectomy.

Compensatory renal growth includes cellular hypertrophy and hyperplasia. The hyperplasia, which occurs mainly in the proximal tubular cells (24,25), is thought to be an early event of compensatory renal growth (1). Furthermore, hyperplasia is age-dependent and is more pronounced in young than in old age (2,3). The increase in renal EGF content which was observed in the early stage of compensatory renal growth was higher in younger than in mature mice. So, according to these results, EGF is more strongly related to hyperplasia than to hypertrophy. In a recent study, Flyvbjerg et al. reported that immunohistoassayable renal IGF-I was increased in the early stage of compensatory renal growth (10). This suggests that IGF-I is also related to the compensatory renal hyperplasia.

This raises the question whether the increase in renal EGF is a result of an increase in renal EGF production or of an increase in renal EGF binding during compensatory renal growth. We therefore examined the renal EGF content immunohistochemically and found that EGF was present at the distal tubular cells and the staining intensity of EGF was increased after uninephrectomy (Fig. 6). It has already been reported that the cortical distal tubular cell is the main site of renal EGF production (21,26,27) and our immunohistochemical findings suggest that the increase in renal EGF content after uninephrectomy is a result of either an increase in renal EGF production or an increased capacity of renal distal tubular cells to store EGF in spite of its release into the urine. It is probable that the increase in renal EGF content in the distal tubular cells after uninephrectomy induces hyperplasia of the neighbouring proximal tubular cells via an extratubular pathway. Further studies are necessary to elucidate further the role of EGF in compensatory renal growth.

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