Virchows Arch (1996) 427:583–588

ORIGINAL ARTICLE

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Expression of epidermal growth factor and its receptor in rabbits with ischaemic acute renal failure

Received: 4 April 1995 / Accepted: 27 October 1995

Abstract Urinary immunoreactive epidermal growth factor (EGF) levels decrease, and renal immunoreactive EGF levels increase in rats with ischaemic acute renal failure (ARF). We investigated the immunohistochemical localization of EGF and EGF receptor in rabbits with ischaemic ARF to clarify the significance of renal EGF. Male New Zealand White rabbits underwent right nephrectomy prior to a 60 min renal artery clamp. At 3, 6, 24, 48, 72 and 96 h after ischaemia, serum urea nitrogen and serum creatinine were determined. Guinea pig anti-rabbit EGF antibody and monoclonal anti-EGF receptor antibody were used for the primary incubation. EGF was immunolocalized to the ascending limb of Henle and the distal convoluted tubule in the normal right kidneys. However, in the post ischaemic left kidneys at 6, 24, 48 and 72 h, immunoreactivity of EGF was associated with proximal tubules. In the normal kidneys, antibody to EGF receptor reacted with distal tubules and collecting ducts. In the ischaemic kidneys, EGF receptor was localized in the basolateral membrane in the proximal tubules. The expression of EGF and EGF receptor in renal tubules may play an important role in repair following ischaemic renal damage.

Key words Epidermal growth factor · Epidermal growth factor receptor · Immunohistochemistry · Acute renal failure · Proximal tubules

Introduction

Epidermal growth factor (EGF), a 53-amino acid polypeptide, was first isolated from the mouse submandibular gland [5], and it is known to have several biological effects, including stimulation of cell mitotic activity and differentiation [4]. Immunoreactive EGF has been identified in extracts of several tissues, including the thyroid glands, pancreas, submandibular glands, duodenum, jejunum and kidneys [12], and in humans, high concentrations are found in the urine [8, 31].

An in situ hybridization study has shown that prepro-EGF mRNA is produced mainly in the distal tubules of the mouse kidney [23], and EGF metabolism and physiology have been investigated in the normal kidney [9], but the role of EGF and its metabolism and secretion in renal failure are unknown. Renal tubular regeneration has been characterized qualitatively in experimental acute renal failure (ARF) [7], and it is clear that EGF is one of the most potent mitogenic stimuli of renal proximal tubular cells in primary culture [14, 20]. Urinary human EGF levels are significantly lower in patients with ARF than in individuals with normal renal function and return to normal after recovery from the acute phase [32].

Urinary immunoreactive (IR) EGF levels have been shown to decrease and renal IR-EGF levels to increase in rats with mercuric chloride-induced ARF [33], but the distribution of EGF and EGF receptor (EGF-R) after ischaemic injury has not been studied. We therefore investigated the effect of ischaemia on expression of EGF and EGF-R in the rabbit kidney.

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Materials and methods

We used male New Zealand White rabbits (Nihon ikagaku doubutu, Tokyo, Japan), weighing 2.5–3.0 kg, fed a standard diet and tap water daily. Animals were deprived of food the night before surgery and body weight and rectal temperature were recorded. They were anaesthetized with isoflurane/oxygen, and surgery was performed under sterile conditions. During surgery, animals were kept on a heated water-recirculating pad and body temperature was monitored. The abdomen was opened via a midline incision and an aortic catheter was inserted to monitor blood pressure and obtain blood samples. The catheter was threaded subcutaneously so that it protruded just posterior to the right scapula. The tip of the catheter was posterior to the ostia of the renal arteries. The

right kidney was removed. Slices of the kidney were frozen in isopentane over liquid nitrogen. The remainder was fixed by immersion in buffered neutral formalin. A 3-ml blood sample was obtained via the aortic catheter for measurement of serum urea nitrogen (SUN) and creatinine [10, 34]. The left renal artery was isolated and occluded for 60 min with a bulldog clamp with the jaws sleeved in silastic tubing. After 60 min the clamp was removed, and the animal was allowed to recover. Control animals (sham-operated) were treated in an identical manner without occlusion of the artery.

At 3, 6, 24, 48 and 96 h after ischaemia, animals were killed. Slices of the left kidney were removed and quickly frozen in isopentane precooled with liquid nitrogen for immunoperoxidase staining. Five minutes before animals were killed, a 3-ml blood sample was obtained. Serum was collected by centrifugation and stored at −20° C for determination of SUN and creatinine levels.

For the immunohistochemical study of EGF the kidneys were homogenized in ice-cold 50 mM acetic acid by a polytron homogenizer (Kinematica, Switzerland). The crude extract was centrifuged at 3000 rpm for 30 min at 4° C. The supernatant solution was evaporated under reduced pressure and then redissolved in a standard diluent for radioimmunoassay (RIA). The rabbit EGF concentration was determined by RIA [36]. Rabbit EGF-specific RIA has been developed using recombinant rabbit EGF [17, 22]. We prepared ^{125}I-labelled recombinant rabbit EGF by the chloramine T method. The standard diluent for the RIA consisted of a 50 mM sodium phosphate buffer (pH 7.4) containing 25 mM EDTA, 140 mM NaCl, 0.5% bovine serum albumin and 0.02% sodium azide. A 100-µl amount of the test sample or recombinant rabbit EGF was mixed in an assay tube with 100 µl of 30000-fold diluted anti-rabbit EGF guinea pig serum, 100 µl of ^{125}I-rabbit EGF and 400 µl of the standard diluent. The mixture was then incubated at 4° C for 48 h. After incubation, 100 µl of normal guinea pig serum, 100 µl of anti-guinea pig IgG goat serum and 200 µl of 15% polyethylene glycol were added to each tube, which was then incubated at room temperature for 1 h. After the sample was centrifuged at 3000 rpm for 30 min at 4° C, the radioactivity in the precipitate was measured with a gamma counter (Aloka, Japan). The intra-assay variation for the RIA was 3.1 to 6.7% and the inter-assay variation was 2.6–6.8%. The minimum detectable concentration was 2 fmol/tube (10 pg/tube).

Immunoperoxidase staining was carried out on frozen cryostat sections cut at 6 µm, fixed in acetone for 10 min at room temperature and air-dried for 5 min. The sections were rehydrated with phosphate-buffered saline (PBS, pH 7.4) and pretreated for 20 min with 20% normal goat serum (Vector Lab, Burlingame, Calif., USA) in PBS to prevent non-specific adsorption. Thereafter, the sections were incubated for 120 min at room temperature in presence of guinea pig polyclonal anti-rabbit EGF antibody (1:1500 dilution in PBS). After several rinses in PBS, the sections were incubated for 60 min with peroxidase-conjugated AffiniPure Goat Anti-Guinea Pig IgG (H+L) (Jackson ImmunoResearch Lab, West Grove, Pa., USA) (1:30 dilution in PBS). The excess of labelled antibody was eliminated by rinses in PBS. Peroxidase staining was performed for 10–15 min using a solution of 6 mg 3,3′-diaminobenzidine tetrahydrochloride (DAB) in 10 ml of PBS containing 100 µl of 3% hydrogen peroxide. The sections were counterstained with Mayer's haematoxylin solution (Sigma, St. Louis, Mo., USA). They were then dehydrated by passing through a graded series of alcohol solutions, mounted with Protexx (Fisher Scientific, Fair Lawn, N. J., USA), and examined and photographed with a photomicroscope.

The polyclonal anti-rabbit EGF antibody was raised in guinea pigs injected with recombinant rabbit EGF, and its specificity has been confirmed by radioimmunoassay methods (×150000). Highly purified rabbit EGF was prepared from the culture supernatant of genetic-engineered *Escherichia coli* in accordance with the method of rat EGF [22]. The amino acid composition and sequence of the recombinant rabbit EGF was identical to that of rabbit urine [17]. Antiserum against rabbit EGF (KFG-103) was prepared by immunizing a guinea pig five times biweekly with an intradermal injection of a mixture of 25 µg recombinant rabbit EGF, 50% polyvinylpyrrolidone and complete Freund's adjuvant. Cross-reactivities in radioimmunoassay with use of the rabbit antiserum to human and rat EGF were 0.06% and 4.3% respectively. Other peptides such as mouse EGF, human transforming growth factor-α (TGF-α), human insulin, human ACTH and human C-peptide did not cross-react.

Quantitative analysis was performed on 3-µm paraffin sections staining with haematoxylin and eosin and studied by light microscopy. They were ranked or graded for tubular necrosis and interstitial inflammation without knowledge of group assignment. Morphological changes were assessed blindly and quantified by ranking as previously described [2, 29]. Severity of tubular necrosis and cast formation was also ranked. Upon completion of the ranking of all specimens, the code was broken and results per group were compared. The tubular localization of EGF was classified into four patterns and compared with the necrosis ranking. To demonstrate the localization of EGF relative to proximal tubular cells, staining for brush border was performed on serial sections. The tubular expression of EGF was compared with the severity of tubular injury.

EGF-R immunocytochemistry was carried out on frozen kidney sections fixed in acetone for 10 min at room temperature and then incubated with 6 M urea/0.1 M glycine HCl buffer (pH 3.5) for 1 h at 4° C. Sections were incubated overnight in a humid chamber at 4° C with mouse monoclonal anti-EGF-R antibody (Transformation Research, Framingham, Mass., USA) (1:100 dilution in PBS). After being rinsed several times in PBS, the sections were incubated for 60 min with peroxidase-conjugated AffiniPure goat anti-mouse IgG (H+L) (Bethyl Laboratories, Tex., USA) (1:100 dilution in PBS). Peroxidase staining was performed for 10 to 15 min using a 6 mg solution of DAB in 10 ml of PBS containing 100 µl of 3% hydrogen peroxide. The sections were counterstained with a methyl green solution, dehydrated by passing through a graded series of alcohol solutions. Negative control included primary incubation with 20% normal mouse serum.

Data are presented as mean±SEM. Statistical analysis was performed by Student's *t*-test. $P<0.05$ was considered to be significant.

Results

The experimental design of this study produced a model of reversible ischaemic ARF, as demonstrated in Table 1. Body weight of animals decreased at 96 h after ischaemic injury, compared with preclamp values. Three hours after clamping, the body temperature of animals was significantly decreased compared with preclamp values, and the drop continued for as long as 48 h after ischaemia. The decrease in haematocrit was apparent at 6 h after ischaemia compared with preclamp values. The haematocrit was at its lowest level at 72 h after clamping. Renal excretory function declined quickly after ischaemic injury as reflected by SUN levels rising in the first 3 h of reperfusion. SUN levels peaked at 72 h after ischaemic insult, and then improved progressively. Serum creatinine levels peaked at 72 h after ischaemic injury.

Immunoreactive EGF levels in rabbit with ischaemic ARF are presented in Table 2. Twenty four hours after ischaemic injury immunochemical study of urinary EGF showed a decrease in urinary EGF level. Immunochemical study of whole kidney tissue showed an increase renal EGF level.

The immunocytochemical procedure clearly demonstrated the presence of anti-EGF reactive material within epithelial cells of renal tubules. In the normal right kidneys, EGF immunoreactivity was mostly confined to the ascending limb of Henle and the distal convoluted tu-

Table 1 Renal function (mean±SEM) following renal artery clamping (*Body Wt* body weight, *Body Tem* body temperature, *Hct* haematocrit, *SUN* serum urea nitrogen, *S-Cr* serum creatinine)

Time, (h)	*n*	Body Wt, kg	Body Tem, °C	Hct, %	SUN, mg/dl	S-Cr, mg/dl
Pre	40	2.76±0.05	38.68±0.08	41.96±0.48	21.9±0.8	1.5±0.1
3	7	3.08±0.13	37.73±0.15*	41.50±1.57	32.1±1.9**	3.0±0.1*
6	7	2.87±0.11	37.98±0.28**	38.92±0.74***	37.7±2.3	3.4±0.2*
24	7	2.59±0.09	37.80±0.64**	38.57±1.53***	72.2±6.6*	5.8±0.9*
48	7	2.64±0.08	38.22±0.24***	34.50±1.00*	110.0±5.0*	7.9±0.7*
72	5	2.67±0.08	38.32±0.36	32.90±0.19*	147.6±28.6*	9.1±1.7*
96	7	2.18±0.08*	38.99±0.16	34.07±0.66*	39.1±7.7**	1.6±0.2

* $P<0.001$ compared with preclamp values; ** $P<0.01$ compared with preclamp values; *** $P<0.05$ compared with preclamp values

Table 2 Immunoreactive epidermal growth factor levels (mean± SEM) in rabbits ($n=5$) with ischaemic acute renal failure (*IR-rEGF* immunoreactive rabbit epidermal growth factor)

Time (h)	Urinary IR-rEGF (ng/mg creatinine)	Renal IR-rEGF (ng/mg wet weight tissue)
Pre	136.84±35.79	1.21±0.25
24	81.83±41.48*	8.38±3.75*

* $P<0.05$ compared with pre-ARF values

Table 3 Evaluation of histology. Histological changes (tubular necrosis, tubular dilation, interstitial oedema) were ranked independently for each slide. The rankings are combined and the values are expressed as mean±SEM. The intensity of the immunoreactivity for anti-EGF antibody on renal proximal tubular cells in rabbit kidneys is graded as negative (–), weakly positive (±), moderately positive (1+), strongly positive (2+)

Time (h)	Necrosis ranking	EGF immunoreactivity in proximal tubules
Preclamp	0	–
3	13.1±3.3	±
6	20.6±2.0	1+
24	25.1±3.1	2+
48	30.9±2.2	1+
72	27.6±6.9	1+
96	7.7±3.5	–

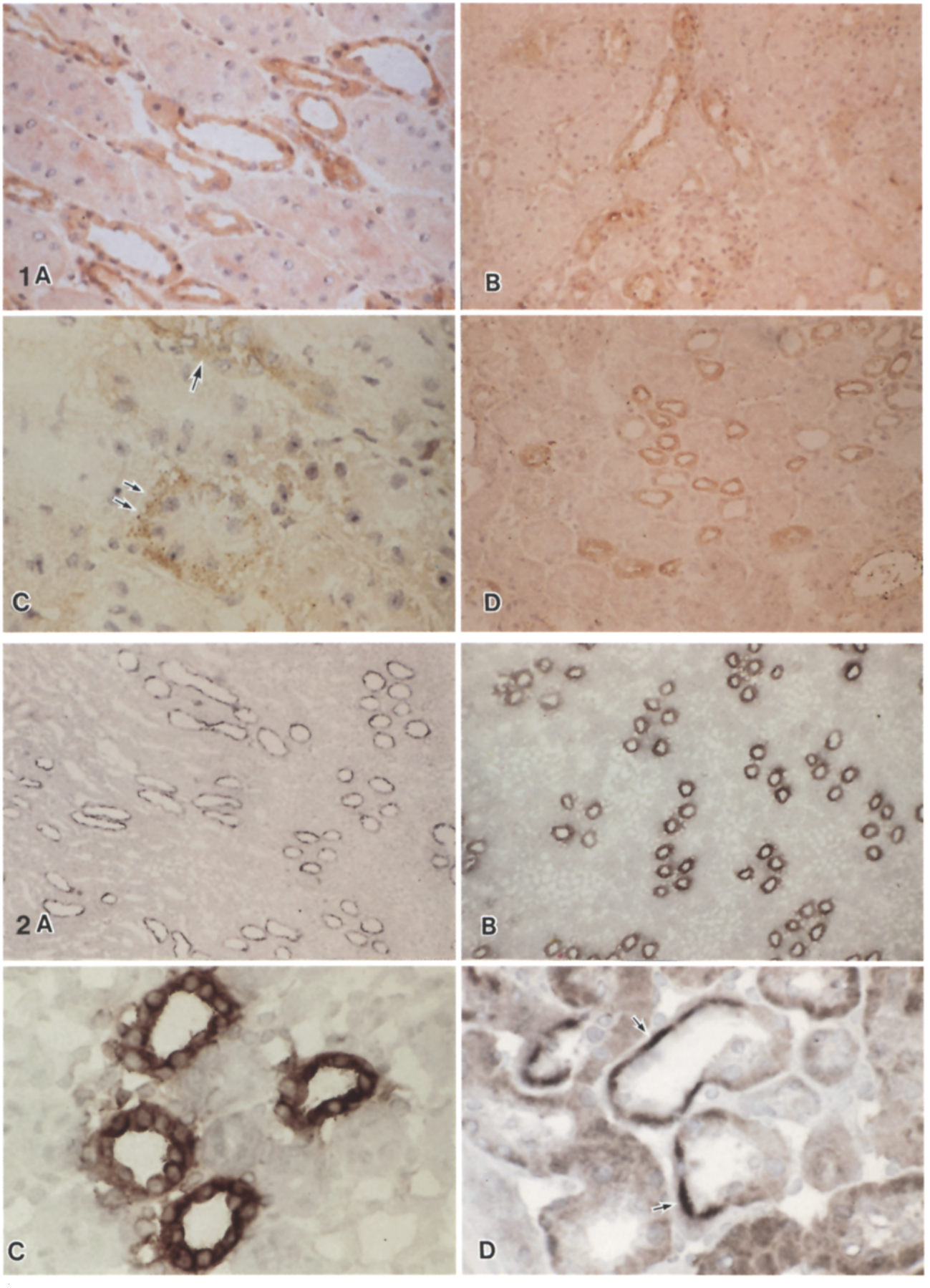
bules (Fig. 1A). At 3 h after ischaemic injury, proximal tubules located in the renal cortex exhibited a weak EGF positive reaction after immunocytochemical staining (Fig. 1B). At 6, 48 and 72 h following ischaemic injury, proximal tubules in the renal cortex exhibited a moderate positive reaction of EGF after the immunoperoxidase procedure. At 24 h after ischaemia, EGF immunoreactivity in proximal tubules was strongly positive (Fig. 1C). At 96 h after ischaemic injury, EGF immunoreactivity was confined to the ascending limb of Henle and distal convoluted tubules in the ischaemic left kidneys (Fig. 1D). Necrosis ranking and EGF immunoreactivity in proximal tubules are summarized in Table 3. Significant necrosis was present at 6 to 72 h after ischaemic injury. Necrosis peaked at 48 h after ischaemia. EGF immunoreactivity in proximal tubules was also significantly positive at this same time period. In the post ischaemic left kidneys at 6, 24, 48 and 72 h, EGF immunoreactivity was associated with proximal tubules, possibly as a response to tubular necrosis.

In the normal kidneys, antibody to EGF-R reacted with collecting ducts and cortical convoluted tubules (Fig. 2A). Cytoplasmic staining for EGF-R was demonstrated in the epithelial cells of distal tubules and collecting ducts in the normal kidneys, however, this staining was more prominent after ischaemia (Fig. 2B, C). Furthermore, in the ischaemic kidneys positive staining for EGF-R was identified in the basolateral membrane of proximal tubules (Fig. 2D). There was no staining in the negative control study.

Discussion

Immunohistochemistry has shown that ischaemic ARF induces expression of EGF and EGF-R in rabbit kidneys. Renal tubular epithelial injury is usually reversible with functional recovery occurring as the tubular cells regenerate and as missing and/or necrotic cells are replaced. In an experimental model of ARF, tubular cell regeneration begins at 24 to 48 h and peaks at 72 h [13], and growth factors that stimulate or augment this regenerating process may lessen the severity and shorten the course of ARF. Urine contains high concentrations of EGF, which has a broad range of biological actions, including effects on tubular cells [8, 31]. In the mouse, prepro-EGF mRNA and EGF are localized exclusively in the thick ascending limb of Henle and the distal convoluted tubules [26].

It has been reported that nephrotoxic and ischaemic renal cell injury reduced prepro-EGF mRNA in renal cortical tissue and urinary EGF excretion [25]. In humans, the kidney expresses high levels of prepro-EGF mRNA [16], but urinary EGF levels in patients with ARF induced by various causes are significantly lower than in normal adults; EGF levels return to normal after recovery [32]. Urinary IR-EGF levels decrease and renal IR-EGF levels increase in rats with experimental ARF [33]. The mechanisms of diminished prepro-EGF mRNA and increased renal EGF are unknown. Urinary human EGF levels are significantly and positively correlated with Ccr and negatively correlated with serum creatinine concentrations [18, 19, 32]; thus, the decrease of glomerular filtration rate induced by ARF may be one of the mechanisms. EGF is stored in the kidney membranes and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release from membrane-associated EGF precursors to promote cell growth following ischaemic ARF [27]. This finding supports our observation and may be one of the reasons for the rise in renal EGF. Earlier reports have documented decreased renal preproEGF mRNA, decreased EGF excretion and increased renal EGF receptors [24, 25]. The increase in renal EGF after renal injury may be due to increased receptor binding of circulating EGF, which would also explain the effect of exogenous EGF.

EGF immunoreactivity in proximal tubules has been observed after aminoglycoside-induced acute tubular necrosis [35]. It is possible that this finding is related to the interaction between endogenous EGF and receptors present on the surface of proximal tubular cells. In the present study, EGF was localized to the ascending limb of Henle and convoluted tubules in normal rabbit kidneys. EGF immunostaining increased in the proximal tubules in response to necrosis, suggesting that expression of EGF may contribute to the repair process following ischaemic ARF. EGF is a potent mediator in repair of tubular injury associated with ARF. It has been reported that the peak in soluble EGF precedes the peak in tubular regeneration, which is consistent with the hypothesis that EGF is one of the mitogenic signals that triggers regenerative hyperplasia after renal injury [28]. These findings suggest that EGF may accelerate renal tubular repair in the acute phase of ARF.

Exogenous administration of EGF early in the recovery phase of ischaemic ARF accelerates epithelial cell replication and shortens the time required for recovery of renal function [13, 21]. EGF and TGF-α have been found to be the most potent mitogenic stimuli of renal proximal tubular cells in culture [14]. The presence of prepro-EGF mRNA in the thick ascending limb of Henle and distal convoluted tubules suggests that there may be an autocrine pathway for EGF release after renal injury, promoting tubular cell replication. A paracrine pathway for EGF release may also be initiated after renal tubule cell injury, promoting proximal tubular cell regeneration. In the present study, excretion of EGF in rabbits with ischaemic ARF was impaired. Local paracrine production of EGF continues in ARF but is inadequate for the task of stimulating tubular regeneration maximally, perhaps because it cannot diffuse properly from the site of production to the site of proliferating cells. It is also possible that although local EGF levels are elevated to 6- to 7-fold normal, even higher levels are required for maximal tubular regeneration, and this is the reason why exogenous EGF treatment is effective.

The mitogenic activity of EGF is mediated by binding of EGF to cell membrane receptors, followed by internalization of the receptor-ligand complex EGF [6]. Receptors for EGF are present in glomeruli, proximal tubules, and collecting ducts [3, 11, 30]. A binding study demonstrated up-regulation of EGF receptors in kidney tissue 1–2 days after renal ischaemia or folic acid administration [1], and an almost 5-fold increase in renal EGF receptors has been observed within 24 h after renal ischaemia [25]. In humans, EGF-R immunoreactivity has been observed in glomerular capillary walls, peritubular capillaries, epithelial cells of distal tubules and collecting ducts [37]. In the present study, EGF-R was localized in the basolateral membrane of proximal tubules in ischaemic kidneys. EGF-R and renal IR-EGF were found to be closely related after ischaemia. Triiodothyronine is found to enhance EGF receptor gene expression, leading to increased EGF receptor mRNA levels, an increase in the number of EGF receptors on the cell surface of renal proximal tubule cells, and potentiation of the mitogenic response to EGF. These effects may represent a molecular mechanism for thyroid hormone-enhanced recovery of renal function following ARF [15]. These findings suggest that up-regulation of EGF-R may be involved in the repair of tubular cells after ischaemia.

In summary, EGF was localized to the ascending limb of Henle and the distal convoluted tubules in normal kidneys. In the post-ischaemic kidney, EGF immunoreactivity was found in the proximal tubules as a response to necrosis. The intensity of EGF staining in renal tubules was related to tubular epithelial cell regeneration, and positive staining for EGF-R was detected in the basolateral membrane of proximal tubules after ischaemic injury. Expression of EGF and EGF-R in renal tubules may play an important part in the repair process after ischaemic injury.

Fig. 1A–D Immunohistochemical localization of epidermal growth factor (EGF) in rabbit kidneys after ischaemic injury. **A** Normal renal section (×400). **B–D** Ischaemic renal sections were obtained at 3 h (**B** ×160), 24 h (**C** ×400), 96 h (**D** ×160) after reperfusion. Sections were incubated with anti-rabbit EGF polyclonal antibody. Immunoreactivity against anti-EGF antibody was observed in the ascending limb of Henle and the distal convoluted tubules in normal right kidneys. Immunoreactivity against anti-EGF antibody was observed in both the distal (*arrow*) and proximal tubular cells (*double arrows*) in ischaemic left kidneys (**C**). At 96 h after ischaemic injury, EGF immunoreactivity was confined to the ascending limb of Henle and distal convoluted tubules in the ischaemic kidneys (**D**)

Fig. 2A–D Immunohistochemical localization of EGF receptor in rabbit kidneys after ischaemic injury. **A** Normal renal section. **B–D** Ischaemic renal sections were obtained at 24 h after reperfusion. Sections were incubated with anti-EGF receptor monoclonal antibody. Immunoreactivity against anti-EGF receptor was observed in collecting ducts and cortical convoluted tubules in normal right kidneys (**A** ×100). Immunoreactivity against anti-EGF receptor was observed in the cytoplasm of epithelial cells of distal tubules and collecting ducts in normal right kidneys, however this staining was more prominent after ischaemia (**B** ×100; **C** ×600). Immunoreactivity against anti-EGF receptor antibody was observed in the cytoplasm of epithelial cells of proximal tubules, especially at basolateral side (*arrows*, **D** ×600)

Acknowledgements This work was partly presented at the 26th Annual Meeting of the American Society of Nephrology, Boston, November 1993, and has been published in abstract form (J Am Soc Nephrol 4:745, 1993). The authors gratefully acknowledge Professor Dr. Kim Solez and Elizabeth A. Monckton (Department of Laboratory Medicine and Pathology, University of Alberta) for their helpful comments and suggestions.

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