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Expression of epidermal growth factor and its receptor in normal and diseased human kidney: An immunohistochemical and *in situ* hybridization study

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Expression of epidermal growth factor and its receptor in normal and diseased human kidney: An immunohistochemical and *in situ* hybridization study. The kidney is one of the major sites of EGF production and there it seems to play several biological functions, such as modulation of cell growth, renal repair following injury, regulation of cellular metabolism and glomerular haemodinamics. The present study was first aimed at localizing EGF and its receptor (R) in normal human kidney by immunohistochemical and *in situ* hybridization techniques. Then, the distribution of the growth factor and its R was explored in biopsy specimens from eight patients with acute tubulointerstitial damage. In the normal human kidney, both EGF immunoreactivity and EGF mRNA were localized in tubular profiles corresponding to Henle's loop and, although to a lesser intensity, to distal convoluted tubule. EGF immunostaining was remarkable mainly at the apical surface of tubular cells. EGF-R protein expression was detected in glomerular endothelial cells, in peritubular capillaries and arteriolar walls, as well as along the thick ascending limb of Henle's loop and distal convoluted tubule, where it colocalized with Tamm-Horsfall protein. Immunohistochemical analysis of tubular profiles revealed that EGF-R was located especially along the basolateral membrane of tubular cells and within the basal part of cytoplasm. Endogenous alkaline phosphatase and CHIP28 positive tubules did not show any signal for EGF and its receptor. Kidneys with acute tubulointerstitial injury exhibited a dramatic decrease of EGF expression, whereas EGF-R showed only minor modifications. Interestingly, EGF-R was localized to both apical and antiluminal membranes of positive tubular cells. It is concluded that EGF-EGF receptor loop may be relevant in the pathogenesis of acute tubulointerstitial damage and recovery from tubular injury, while its role in the physiological renewal of the urothelium remains speculative.

Epidermal growth factor (EGF) is a 53 amino acid peptide, produced as a large precursor molecule (prepro EGF) which can exist as a biologically active transmembrane protein [1–3]. Current information indicates that the mammalian kidney is a significant site of EGF synthesis, probably exceeding most other tissues in the human species [4]. A long array of renal responses to administration of EGF have recently been reported, including modulation of glomerular hemodinamics, renal metabolism, tubular transport and eicosanoid synthesis [5]. In addition, EGF may direct renal cell proliferation during fetal life [6], and maintain and repair the epithelial lining of ductal system [5].

The EGF receptor is a glycoprotein with tyrosine kinase activity [7], which mediates the biological signal not only of EGF, but also of numerous related peptide growth factors, such as transforming growth factor α (TGF-α) [8], the vaccinia virus growth factor (VGF) [9], amphyregulin [10] and, possibly, tenascin [11]. Binding to the EGF receptor (R) triggers a cascade of events which ultimately regulate the growth of a variety of epithelial and mesenchimal cell types [12].

The primary sites of EGF synthesis in the rat and mouse kidney have been localized to the thick ascending limb of Henle's loop and to distal convoluted tubule [4, 13–16], while specific high affinity EGF receptors have been demonstrated in mesangial cells, proximal tubule, cortical and inner medullary collecting ducts, as well as in medullary interstitial cells [17–20].

Since both the rat and the mouse are commonly used as a model for various human disorders, it is relevant to know if the human kidney shares the same pattern of renal EGF expression. Thus, in the present study we first aimed to map EGF and EGF-R distribution in the human kidney, by using *in situ* hybridization and immunohistochemistry. Since current evidence supports a role of EGF in repair and recovery of ischemic or nephrotoxic experimental acute renal failure, we subsequently evaluated the gene and protein expression of EGF loop in patients with acute tubulointerstitial injury.

Methods

Tissue

Eight normal-appearing kidney portions obtained from patients undergoing nephrectomy for renal carcinoma were immediately included in saline, snap-frozen and stored in liquid nitrogen until used. Moreover, biopsies of eight patients affected by acute tubulointerstitial nephritis were also studied. Frozen sections (6 μm thick) were collected onto polylisine-coated slides, dried briefly on a hot plate at +80°C and fixed in 4% paraformaldehyde for 20 minutes. After two washes in PBS, dehydration in graded ethanols and short air drying, sections were immediately used for *in situ* hybridization. For immunohistochemical studies, frozen

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Table 1. Plasmids used for the preparation of probes

Plasmid	Species	Gene fragment size	Predominant region encoded
Lambda EGF-116	human, EGF	Eco RI-Bam HI 1000 bp	5′ coding sequence
pHER-A64-1	human, EGF-R	Eco RI-BAM HI 900 bp	part of the 3′ encoding sequence

sections (4 μm thick) were air dried overnight and stored at −80°C. Moreover, to better define the cellular profiles, 2 μm paraffin-embedded sections were also used.

Probes

For the preparation of RNA probes, the cDNA fragments listed in Table 1 were subcloned into the plasmid pGEM1 (Promega Biotec, Florence, Italy) at the appropriate restriction sites. After linearization of the plasmids with either *Hin*dIII or *Eco*RI restriction endonuclease, T7 or SP6 RNA-polymerase (Boehringer Mannheim, Germany), was employed to obtain run off transcripts of either the anti-sense (complementary to mRNA) or sense (anticomplementary, negative control) strands, respectively. Transcription and labeling of RNA probes were performed as described [21]. Briefly, for *in situ* hybridization, 60 μCi of [^{35}S]uridine-5′-(a-thio)-triphosphate (S.A.:1,250 Ci/mmol; Amersham, Little Chalfont, UK) was added to a 10-μl reaction mixture (0.5 mM each of adenosine-, cytidine- and guanosine-5′-triphosphate/1 mM dithiothreitol/10 units of human placental RNase inhibitor/6 mM $MgCl_2$/10 mM Tris-HCl, pH 7.5/2 mM spermidine/10 mM NaCl) including 1 μg of linearized plasmid and 16 units of either SP6 or T7 RNA polymerase. The reaction was allowed to proceed for 60 minutes at 38°C. The plasmid DNA was removed by digestion with 25 μg/ml RNase-free DNase I in a mixture containing 2.5 mg/ml of yeast tRNA and 10 units of RNase inhibitor for 10 minutes at 37°C. Free ribonucleotides were removed by phenol-chloroform extraction followed by ethanol precipitation. To increase the penetration into tissue, the size of the ^{35}S-labeled RNA probes was adjusted to 50 to 200 bases length by a controlled alkaline hydrolysis in 80 mM $NaHCO_3$/120 mM Na_2CO_3, pH 10.2/10 mM dithiothreitol at 60°C. After neutralization in 0.2 M sodium acetate, pH 6.0/1% acetic acid/10 mM dithiothreitol and ethanol precipitation, RNA probes were stored at −80°C and used within four weeks. The specific activity usually obtained was 1.2 to 1.4 × 10^9 cpm/μg of ^{35}S-labeled RNA probe.

In situ *hybridization*

Prehybridization, hybridization, removal of nonspecifically bound probe by RNase A digestion and further washing procedures for both positive and negative strand RNA probes were performed as described previously [21]. Briefly, air-dried sections were treated in 0.2 N HCl for 20 minutes and then washed in H_2O for five minutes. After 10 minutes of digestion with 0.125 mg/ml Pronase (Boehringer Mannheim) in PBS and a quick rinse in 0.1 M glycine/PBS, slides were fixed for 20 minutes in 4% paraformaldehyde/PBS. Sections were then washed in PBS for five minutes and acetylated in a freshly prepared solution of acetic anhydride diluted 1:400 in 0.1 M triethanolamine, pH 8.0, for 10 minutes. After washing in PBS for five minutes, sections were dehydrated in graded ethanols and air dried prior to hybridization. Hybridization mixture (50% formamide/10% dextran sulfate/10 mM diothiothreitol/0.1 M Tris-HCl, pH 7.5/0.1 M Na_3PO_4/0.3 M NaCl/50 mM EDTA/1 × Denhardt's solution/0.2 mg/ml yeast tRNA), containing 2 × 10^5 cpm of ^{35}S-labeled RNA probe in a final volume of 25 μl, was applied on each section and covered with a siliconized coverslip. Hybridization was continued for 18 hours at 50°C in a sealed humid chamber. Excess of probe was removed by washing for four hours at 50°C in 0.1 M Tris-HCl, pH 7.5/0.1 M Na_3PO_4/0.3 M NaCl/50 mM EDTA/1 × Denhardt's solution/10 mM dithiothreitol. To decrease background, slides were incubated for 30 minutes at 37°C with 20 μg/ml RNase A in 0.1 M Tris-HCl, pH 7.5/1 mM EDTA/0.5 M NaCl. After 30 minutes wash at 37°C in the same buffer without the enzyme, sections were further rinsed in 2 × SSC/0.1% SDS and 0.1 × SSC/0.1% SDS, 30 minutes each, dehydrated in graded ethanols containing 0.3 M ammonium acetate and finally air dried prior to the autoradiographic procedure.

Autoradiography was performed by dipping the dehydrated slides into Ilford G5 nuclear emulsion (Ilford, Mobberley Cheshire, UK), melted at 42°C and diluted 1:1 in 0.6 M ammonium acetate. After two hours of drying, the slides were stored in light-proof boxes containing dessicant and exposed at 4°C for 7 to 28 days. The exposed slides were developed in Kodak D19 developer (Kodak, Hemel Hampstead, UK) for 2.5 minutes, rinsed in 1% acetic acid and fixed in Kodak Fixer for three minutes. After extensive washing in tap water, the slides were finally counterstained in hematoxylin-eosin and mounted in Corbitt balsam.

Immunohistochemistry

Immunohistochemical detection of EGF and EGF-R was performed on frozen sections using chromatography-purified mouse antibodies specific for human EGF (IgG1, clone 144-8, Oncogene Science, Inc., Manhasset, NY, USA) or EGF-R (IgG2a, clone 528, Oncogene Science, or EGF-R IgG1, clone 29.1, Technogenetics, Milan, Italy), at a dilution of 1:50 to 1:600. To better define the distribution of EGF-R along the cellular profile, paraffin-embedded sections (2 μm thick), pre-treated for 10 minutes at room temperature with a 0.1% solution of ficine (Sigma, Milan, Italy), were also used. Immobilized mouse antibodies were detected by the immunoalkaline phosphatase (APAAP) method with an affinity-purified rabbit anti-mouse immunoglobulin serum (1:20 dilution, DakoPatts, Copenhagen, Denmark) and APAAP complex (1:50 dilution, DakoPatts). Each step was followed by two washes (5 min each) in Tris-hydrochloric acid (HCl) buffered saline (TBS; pH 7.5). Alkaline phosphatase was developed with a mixture of naphtol AS-BI phosphate and new fuchsin [21]. Levamisole (Sigma, Munich, Germany) was added to the development solution in order to block endogenous alkaline phosphatase activity. Finally, sections were weakly counterstained with Mayer's hemalum, and coverslipped using Kayser's gelatin. Negative controls were performed by omitting the primary or secondary antibodies, and employing non-immune mouse serum as the first layer. The identification of tubular segments was performed as follows: (1) for the proximal tubule, endogenous alkaline phosphatase was detected by using a mixture of naphtol AS-BI phosphate and new fuchsin lacking levamisole, and the CHIP28 protein (a proximal tubule water channel) by an indirect immunofluorescence technique employing a rabbit polyclonal anti

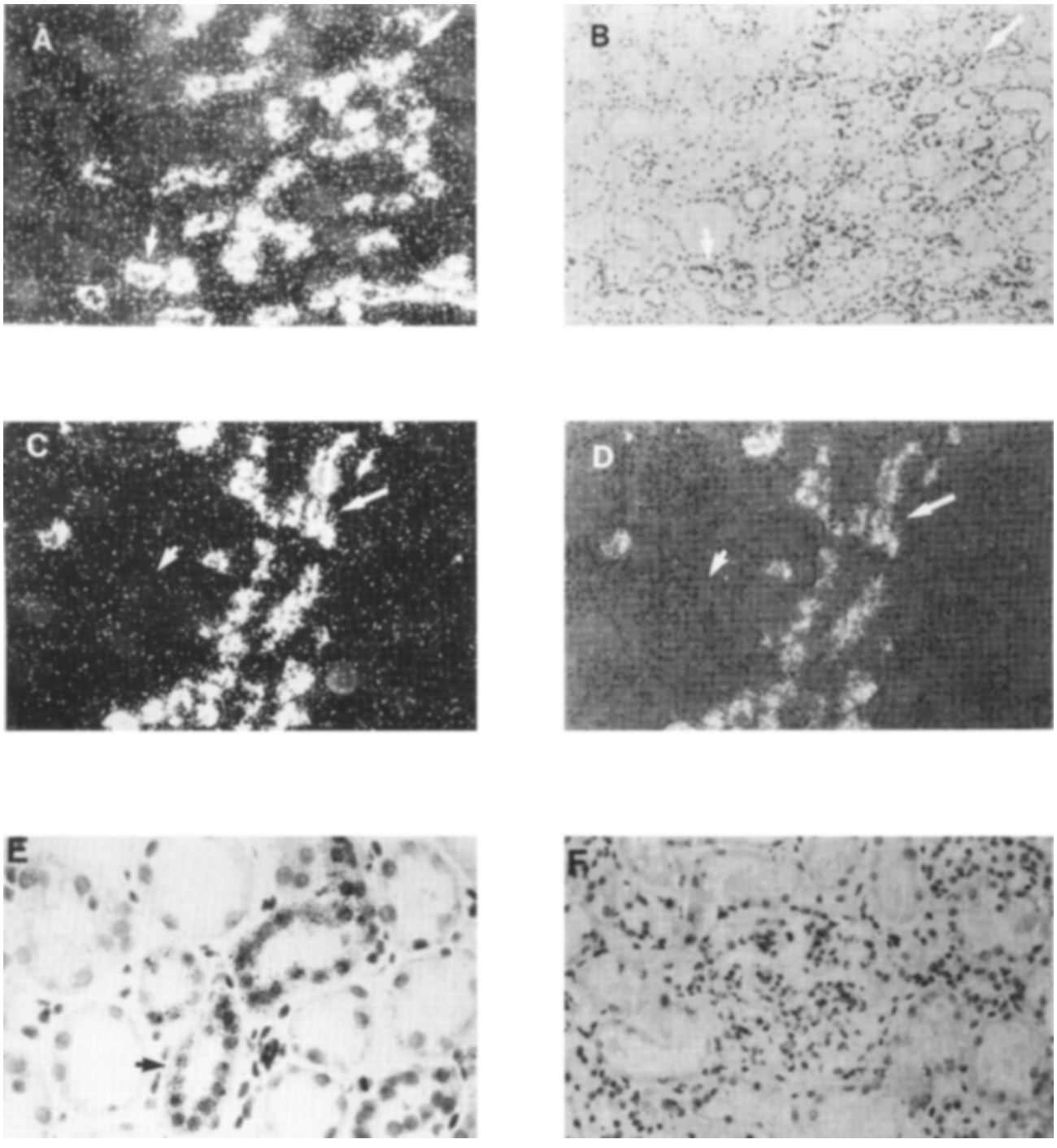

Fig. 1. *EGF mRNA in normal-appearing kidney.* Dark (**A**) and bright (**B**) field photomicrographs of normal-appearing portions of kidney reveal positive hybridization for EGF at the tubular level (×100; positive hybridization is indicated by collection of white or black grains in the dark and bright fields, respectively). Note the striking collection of grains in the distal convoluted tubules (↑) and ascending limbs of Henle (↑) at the medullary level. (**C**) *In situ* hybridization of a section from the same tissue specimen shown in A and B, which demonstrates the lack of cell-associated collection of silver grains at the glomerular level (↑) and the marked positivity of cortical tubular profiles (↑) (×100, dark field photomicrograph). (**D**) A multiexposure dark-bright photomicrograph (×100) of the same field shown in **C.** (**E**) Bright field photomicrograph showing positive hybridization for EGF in the distal convoluted tubules (▲) at a higher magnification (×400; positive hybridization is indicated by collection of black grains). (**F**) Sense probe control. Bright-field microphotograph (×200) of a section from the same specimen shown in **E.** This section was hybridized with sense probe for EGF and developed in parallel with that shown in E. Note the lack of hybridization signal, compared to **E.**

human CHIP28 antibody (donated by Dr. G. Valente) followed by a fluorescein-conjugate anti rabbit antibody [22]. (2) For the ascending portion of Henle's loop and the early distal tubule, a direct immunofluorescence technique was employed using a fluoresceine-conjugate anti-human Tamm-Horsfall protein (provided by Dr. G.B. Fogazzi) [23].

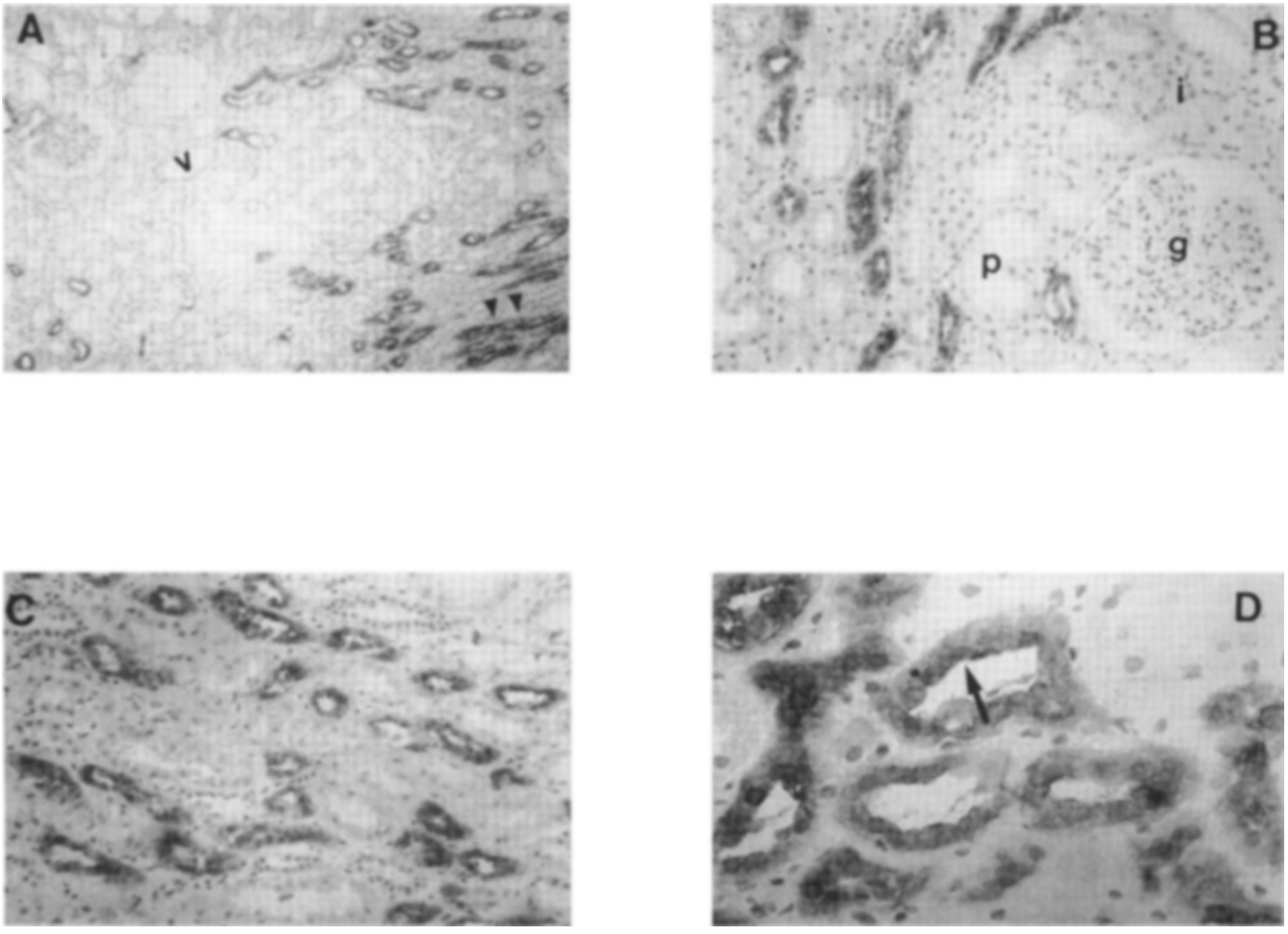

Fig. 2. *(A-D) Immunohistochemistry for EGF protein in normal-appearing kidney.* Immunostaining is seen as the dark red alkaline phosphatase product in the distal convoluted tubules (↑) and ascending limbs of Henle (↑ ↑). Note the striking positivity of the immunohistochemical signal expecially at the apical cellular surface. A scanty positivity, if any, can be observed within the glomerulus (g), while the proximal tubule (p), the renal vessels (v) and the interstitium (i) do not show any immunolocalization. (A ×100; B ×200; C ×200; D ×400).

Results

In situ hybridization of normal-appearing portions of human kidney revealed EGF expression in tubular profiles of both renal cortex and medulla. Specifically, the cortical and the medullary thick ascending limb of Henle (TAL) and the distal convoluted tubule (DCT) showed an intense hybridization signal, whereas cells of the macula densa did not show any hybridization (Fig. 1 A-E). EGF antisense RNA probe did not label any other portion of the nephron, vasculature, or interstitium (Fig. 1 A-E). The lack of hybridization with control "sense" probe (Fig. 1F), along with the lack of detection of silver grains at the renal edge, apparently demonstrates that the hybridization signal detected was highly specific.

Similar patterns of distribution were observed by APAAP staining (Fig. 2 A-D). Protein expression was observed only in TAL and DCT, while the macula densa, the proximal tubule, the renal vessels and the interstitium were constantly negative (Fig. 2 A-D). Positive cells showed an intense immunohistochemical signal, localized mainly at the apical surface. As regards the glomerulus, very scanty, if any, immunohistochemical positivity was observed (Fig. 2B).

In contrast, EGF-R mRNA expression was localized in TAL and DCT, in the glomerulus and interstitial vascular tree (not shown). By immunohistochemistry, TAL and DCT cells displayed a strong signal (Fig. 3A), while the proximal tubule and the interstitial cells resulted negative. A positive immunoreaction for EGF-R was remarkable mainly along the basolateral membrane of tubular cells and in the neighboring cytoplasm, where the remaining cytoplasm exhibited only a scanty positivity (Fig. 3A). At the glomerular level, an intense positivity was detected in endothelial cells, whereas visceral epithelial and mesangial cells were constantly negative (Fig. 3B). Moreover, endothelial cells of peritubular capillaries and arteriolar walls displayed a strong and constant positivity for EGF-R (Fig. 3C). In the Henle's loop and in the early distal tubule, the distribution of EGF-R displayed an almost perfectly matching colocalization with the Tamm-Horsfall protein (Fig. 3 E, F). In control slides, when non-immune mouse serum was used, no immunohistochemical signal was apparent (not shown).

Biopsy specimens from patients with acute renal failure exhibited a marked and diffuse down-regulation of prepro-EGF gene and protein expression (Fig. 4 and 5). Concomitantly, we studied

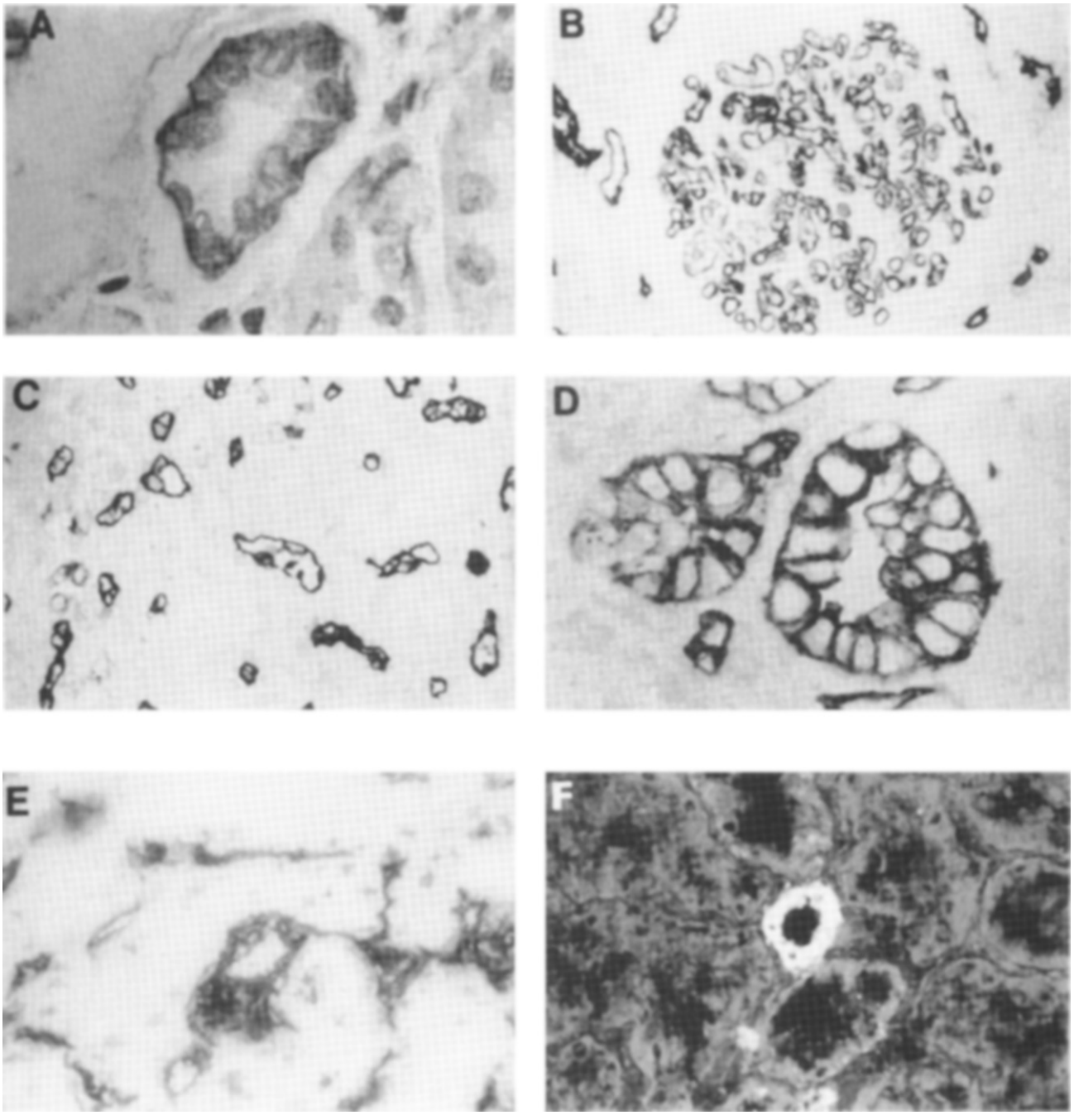

Fig. 3. *Immunohistochemistry for EGF-receptor protein in normal-appearing* (**A-C**) *and diseased kidney* (**D**). In normal kidney EGF receptor immunoreactivity is localized mainly in the basal part of the cytoplasm and along the basolateral membrane of tubular cells (↑ ; A ×1000). Note the intense staining along the endothelial layer within the glomerulus (B × 400) and the interstitial vascular tree (C ×400). In panel **D**, it can be appreciated the redistribution of the receptor in a diseased kidney. **(E-F)** Immunoreactivity for EGF-R (E ×400) and Tamm-Horsfall protein (F ×400) showing an almost perfect matching colocalization at the tubular level (↑).

the gene and protein expression of C3 and monocyte chemotactic protein-1 in the same samples and found a net increase of both signals in the whole proximal tubule (not shown), which seemingly rules out a generalized effect of nephrotoxic injury on mRNA and protein synthesis, confirming the specificity of EGF down-regulation. In contrast, EGF-R expression did not present any major change in the intensity of the signal at the glomerular, tubular and interstitial vascular level. Interestingly, a redistribution of the receptor protein in the Henle's loop and DCT was observed, such that it was seen not only at the basolateral cell surface but also at the apical level (Fig. 3D).

Discussion

The present *in situ* hybridization study has shown that prepro-EGF is expressed in the human kidney and is localized in the Henle's loop and in the distal convoluted tubule. Immunohistochemical staining parallels the distribution of EGF as shown by *in*

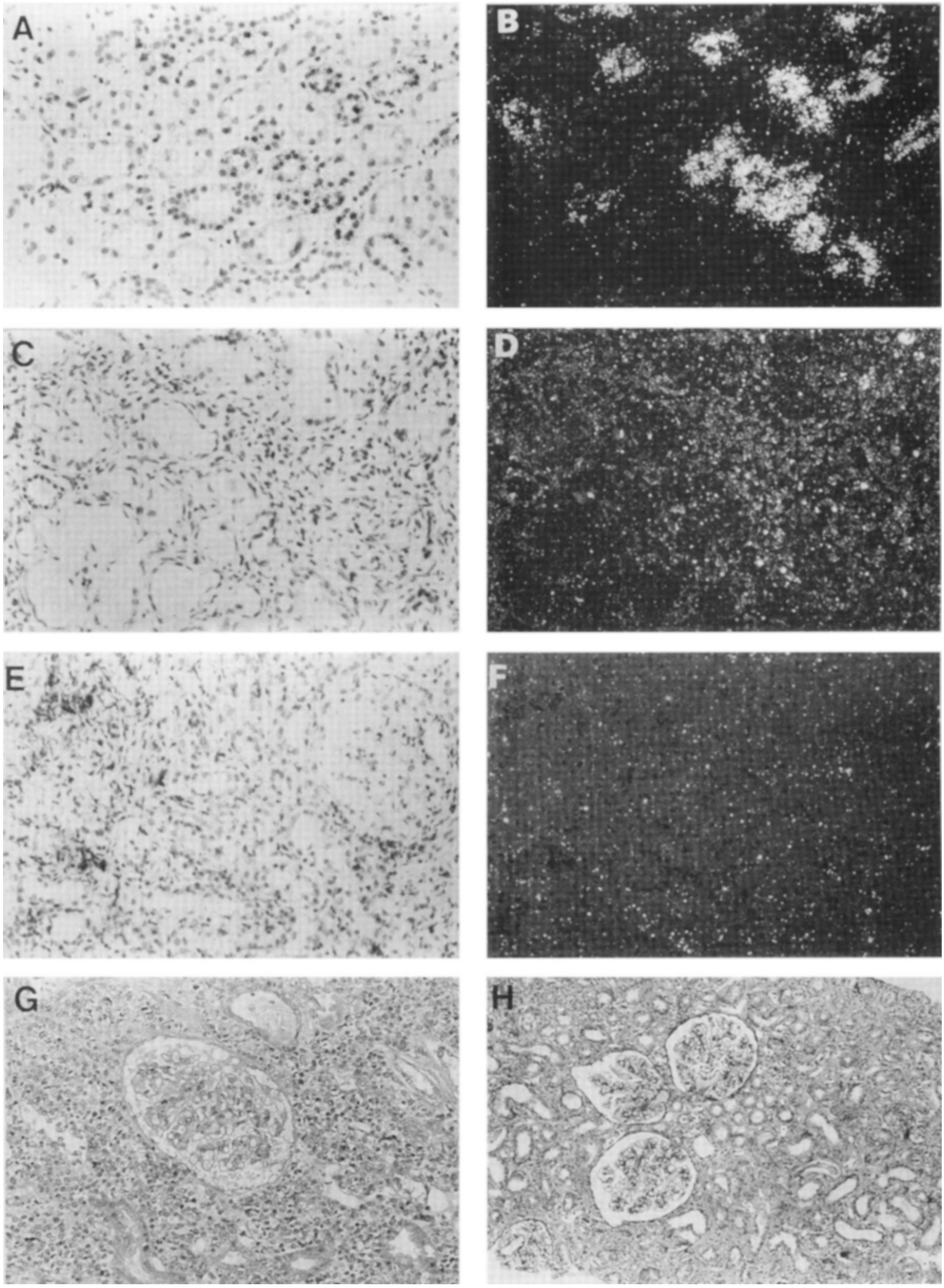

Fig. 4. *Expression of EGF mRNA in normal (***A** *and* **B***) and diseased kidney (***C** *through* **F***).* Note the striking downregulation of EGF hybridization signal in acute tubular damage (**C-F**) compared to normal (A, B). (**G** and **H**) Light microscopy features (PAS staining) observed in paraffin embedded sections obtained from Bouin's included portions of the same kidney showed in panels C-D and panels E-F, respectively. Note the tubular damage, the edema and the presence of infiltrating cells.

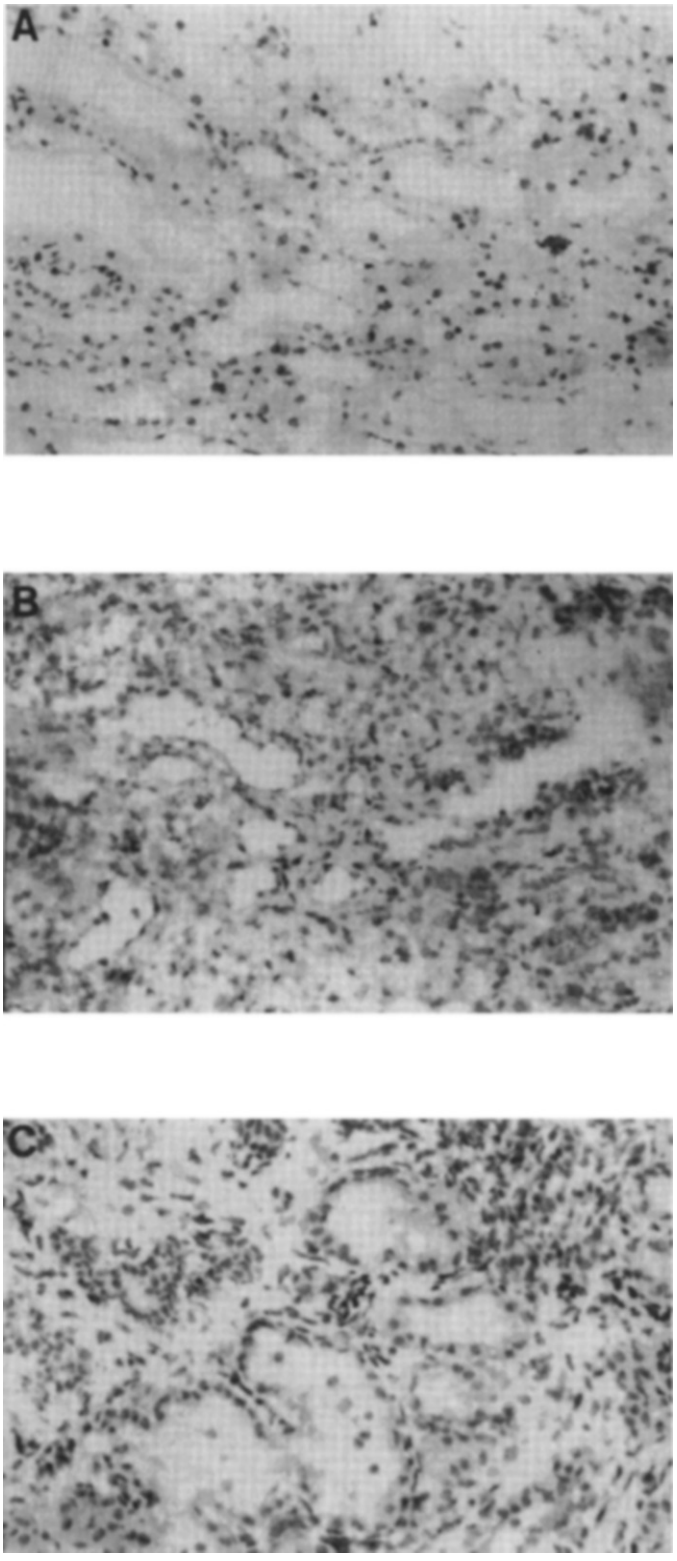

Fig. 5. *Expression of EGF protein in 3 different diseased kidneys (***A-C***).* Note the striking reduction of EGF protein, more prominent in **A** and **C**, compared to normal kidney (Fig. 2).

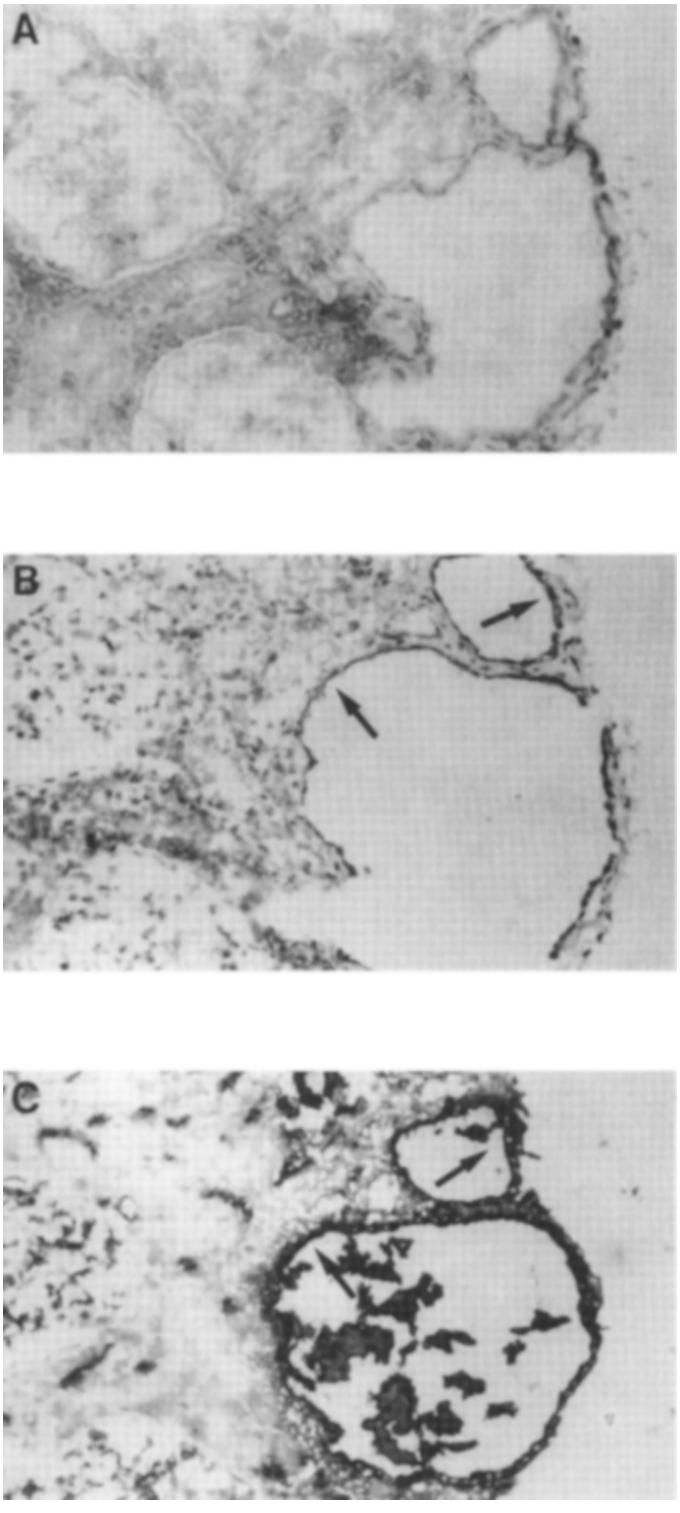

Fig. 6. *(***A***) A serial section of (***B***) and (***C***) incubated with a nonimmune mouse serum.* EGF (B) and EGF-R (C) protein expression in two cystic tubules. Note the presence of EGF in the epithelial layer of the cysts (↑) compared to the negativity in the remainder tubular profiles and the redistribution of EGF-R (↑).

situ hybridization, with very scanty, if any, positivity at the glomerular level.

The pattern of EGF mRNA localization in the human kidney strictly resembles the distribution of the growth factor in the mouse and rat kidney [14–16]. The EGF system in rodent kidney has been extensively studied in experimental models of tubular damage [24–29] and of compensatory renal hypertrophy [30–32]. The results of the present investigation indicate that EGF actions

in these pathophysiological models may be relevant to the human kidney.

Nouwen and De Broe have recently described EGF protein distribution in adult human kidney [33]. They identified two forms of EGF immunoreactivity. One was distributed along the apical cell surfaces and in the cytoplasm of the thick ascending limb and in the distal convoluted tubule. The other was detected as membranous staining in the connecting tubule and cortical collecting duct, and as basal staining in the rest of collecting duct. As far as the second pool of EGF staining is concerned, the authors could not distinguish between the existence of an EGF-related or -resembling epitope or a peculiar conformation of the growth factor, that is masked in native material. We could confirm the distribution of EGF immunostaining along the thick ascending limb and the early distal convoluted tubule, while EGF mRNA localization by *in situ* hybridization demonstrated the local production of the observed immunoreactivity.

Yoshioka et al reported the presence of EGF in human glomeruli, as revealed by both radioimmunoassay of glomerular homogenates and indirect immunofluorescence on serial slices of kidney specimens [34]. Further, ultrastructural studies revealed that EGF was localized to the membrane surface of glomerular endothelial cell, whereas EGF staining was absent in epithelial or mesangial cells of glomeruli [34]. Apart from possible differences in the sensitivity of antibodies used, our *in situ* hybridization results tend to support the view that EGF-positive immunostaining of human glomerular endothelial cells, when present, cannot be ascribed to a local synthesis of the growth factor, but rather to EGF-R uptake (see below).

The mRNA for EGF-R is fairly widespread, particularly in comparison to the focal and discrete pattern of EGF expression. This more generalized pattern of receptor gene expression tends to be true for most tissues [7]. EGF-R localization, both at transcriptional and post-transcriptional level, was detected mainly along the Henle's loop and the distal tubule, in endothelial cells within the glomerulus, as well as in peritubular capillaries and arterioles of the interstitium. A pattern of distribution of EGF-R roughly resembling that described here had been depicted in two previous immunohistochemical studies of the human kidney [34, 35]. In addition to its growth promoting activity, EGF possesses a number of other biological activities, including stimulation of arachidonate metabolism [36] and vasoconstriction of vascular smooth muscle [37]. The localization of EGF receptors within the arteriolar walls and along glomerular and peritubular capillaries supports the potential for EGF to act as a regulator of microcirculatory dynamics within the human kidney.

In the kidney, recovery from ischemic or nephrotoxic tubular necrosis requires a regenerative response eventually leading to re-epithelialization of damaged tubules. Current evidence from experimental models points to the importance of EGF in the regulation of cell proliferation and differentiation following acute renal injury. We therefore studied renal expression of EGF and its R in patients with acute tubulointerstitial damage and revealed a drastic decrease of EGF mRNA and protein expression, whereas EGF-R showed minor modifications, and presented chiefly a cellular redistribution. After acute experimental tubular injury, expression of prepro-EGF mRNA and excretion of EGF by the kidney fall dramatically [26, 38, 39]. The decline in the amount of mRNA encoding the EGF precursor supports the conclusion that the modification of EGF immunolabeling reflects depressed synthesis of the protein. On the other hand, Schaudies and Johnson revealed that post-ischemic tubular injury involves an enzymatic conversion of EGF precursor into diffusible, low molecular weight, EGF [40], whose release would take place before the actual onset of regenerative hyperplasia [41]. In our opinion, these findings may provide a molecular basis for a possible paracrine effect of EGF during tubular regeneration. Indeed, *in vivo* administration of EGF promotes tubular cells proliferation and accelerates the recovery of renal function in rat acute renal failure [24–27].

Up-regulation of EGF receptors has been reported to occur in rat kidney after induction of ischemia in folic acid intoxication [42]. In contrast, Toubeau and coworkers were unable to show any major change of EGF-R immunostaining in rat tubular epithelium during tubular necrosis, but described the complete disappearance of the signal in tubular profiles undergoing regenerative hyperplasia [43].

Studies describing EGF loop localization in patients with acute renal failures are presently lacking. Nakopoulou et al evaluated the expression and distribution of EGF-R in various types of human renal injury and described a higher, although not statistically significant, expression of the protein in patients with membranous glomerulonephritis and in focal segmental glomerulosclerosis, whereas renal grafts with acute rejection, which may resemble the pattern evoked by acute tubulointerstitial injury, did not show any modification when compared with normal kidney [35].

Occupied EGF receptors are internalized and transferred to the lysosomal compartment, where EGF-R complexes undergo proteolytic degradation, a process which eventually leads to surface R down-regulation. Moreover, EGF appears to increase mRNA encoding its own receptor in some cellular systems [44]. Thus, it may be inferred that the decrease of EGF protein, observed in acute tubulointerstitial injury, implies the disappearance of a specific signal leading to receptor synthesis and/or degradation.

In the normal human kidney, prepro-EGF is localized to the apical surface of tubular cells, whereas EGF receptors have an exclusively basolateral localization, a distribution which comes true also in animal kidney [14–16, 45, 46]. Investigations characterizing EGF action in isolated-perfused collecting tubules have shown that the peptide is active only when exposed to the antiluminal membrane of tubular segments [47–48]. Therefore, a paracrine interaction between renal EGF and its receptor appears questionable. Indeed, Nouwen and De Broe [33] presented data indicating a basal localization of EGF precursor or a EGF-related substance in the connecting tubule and collecting duct of human kidney, which would make a juxtacrine interaction possible, although limited to the above segments. In this setting, the redistribution of EGF-R immunostaining within renal tubular cells, such that the protein is present at both the luminal and basolateral locations, is particularly interesting (Fig. 3D). Following uninephrectomy of rats, immunostainable EGF in controlateral kidneys appears localized adjacent to both luminal and antiluminal membranes [32]. Moreover, an apical distribution of both EGF-R and prepro-EGF in cells lining collecting tubule cysts of human autosomal recessive polycystic kidney disease has been recently described [49, 50]. We ourselves found a similar redistribution of EGF-R in dilated tubules as well as the presence of EGF (Fig. 6). Some glomeruli from patients with acute renal failure showed a positive signal for EGF and Tamm-Horsfall protein (with prepro-mRNA being negative), presumably because of a

back diffusion of the proteins within the obstructed tubules (not shown). A similar phenomenon was already described for Tamm-Horsfall protein and might be relevant for the regulation of intraglomerular microcirculation through the binding of released EGF to endothelial EGF-R [23].

Collectively, these observation support a role for the EGF–EGF-R loop in the pathogenesis of acute tubulointerstitial injury and/or in the recovery from renal damage in humans, whereas the autocrine/paracrine function of EGF in the physiologic renewal of the urothelium remains speculative.

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