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Altered growth factor expression during toxic proximal tubular necrosis and regeneration

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Altered growth factor expression during proximal tubular necrosis and regeneration. Growth factor expression was investigated during the regenerative response after toxic proximal tubular necrosis. Therefore, gentamicin was administered to rats to achieve an experimental model, characterized by the appearance of segment-specific proximal tubular necrosis, that is followed by a regenerative response leading to functional and morphological recovery in a limited time. Four days after the administration of the highest dose, serum creatinine rose to a mean value of 5.8 mg/dl and returned to normal values ten days after the treatment. The S1-S2 segment of the proximal tubules in the cortex became clearly affected by severe toxic necrosis one day after the treatment, while maximal necrosis was observed at days 2 to 4. Only minor injuries were noticed in the other renal compartments. The proliferative response started in the interstitial cells first. The major proliferative wave was localized in the convoluted part of the proximal tubules at days 6 to 8, although proliferation was also prominent among non-proximal tubular cells. A profound interstitial infiltration of leukocytes, including macrophages and T lymphocytes, was observed. Ten days after the treatment the functional and morphological recovery were completed. Slot blot hybridization revealed a decreased EGF and IGF-I mRNA expression from the start of the observation period. While IGF-I mRNA had regained its normal expression at day 10, EGF mRNA was still below control levels. The PDGF-B transcript became more abundant towards the end of our observations. No major changes in the expression of TGF-α, TGF-β1 and c-*fos* were detected. Renal EGF-immunoreactivity disappeared from the luminal plasma membrane of the distal tubular cells analogous to the results obtained at the messenger level. However, EGF-staining was lost in the cortex first, hence a topographical association between the loss of EGF-immunoreactivity in the distal tubules and the observed necrotic lesions in the proximal tubules was found. Immunoreactive EGF was never observed in proximal tubular cells from normal, injured or regenerating rat kidneys. We conclude that in this experimental rat model, EGF and IGF-I mRNA expression is decreased during the regenerative response upon severe toxic tubular necrosis. No evidence for a participation of EGF or IGF-I of renal origin in the recovery of the kidney is found.

Acute renal failure caused by toxic substances evokes a proliferative response in the damaged tubules and often also in the interstitium [1]. Although the mechanisms of injury that lead to cell death and the tubular cells that are predominantly affected can be very diverse [2], necrotic tubular cells are thought to be replaced by the remaining cells, which, from their resting state (G_0), re-enter the cell cycle and proceed through DNA-replication (S) into mitosis (M). Currently, the signals that activate this transition are not well understood [3]. However, one can presume that, in order to restore the complete tissue architecture and kidney function, control of this process has to be very precise.

There is increasing evidence that polypeptide growth factors such as epidermal growth factor (EGF), the transforming growth factors (TGF-α and TGF-β), the insulin-like growth factors (IGF-I and IGF-II), the fibroblast growth factors (aFGF and bFGF), plateled-derived growth factor (PDGF) and proto-oncogenes like c-*fos* could be involved in the regenerative processes [4–6]. Indeed, tubular cells as well as interstitial cells can produce and/or secrete several of these factors, and they also bear receptors for them [7]. Hence, the local release of growth factors may modulate the repair process in an autocrine, paracrine, juxtacrine or endocrine manner [8–10]. Our current knowledge about growth factor activities is mainly gathered by *in vitro* research [11]. However, substantially less is known about their physiological relevance *in vivo*. Unravelling the expression pattern of these factors during the regenerative process can provide new insights in their roles in the restoration of the structural and functional integrity of the kidney [12, 13].

In an experimental rat model different doses of gentamicin were used to induce varying degrees of proximal tubular necrosis, mainly confined to the convoluted part (S1-S2 segments). Two experimental designs were used in this study. Since in a preliminary experiment serum creatinine values did not rise, necrotic lesions were absent despite the presence of cellular proliferation, and the EGF mRNA level remained unchanged after administration of 140 mg/kg gentamicin, a substantially higher dose of gentamicin was used in the present study to induce regenerative events in a limited period of time in response to extensive segment-specific tubular necrosis. A second experimental design was inspired by the description of a redistribution of renal EGF after gentamicin or amikacin administration to rats [14, 15].

Growth factor expression was evaluated concomitantly with the damage and subsequent regeneration, which we investigated in the different histological compartments for a period of ten days. As interstitial infiltration is a recognized feature of nephrotoxic injury [1, 2, 16] that was also observed in previous studies of our laboratory [17, 18], and because of its potential importance in tissue remodeling, the identity of the interstitial infiltrating cells was also determined and their presence was quantified. In addition to the mRNA expression of renal EGF,

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TGF-α, TGF-β1, IGF-I, PDGF-B and c-*fos*, the histological distribution of EGF of renal origin was investigated immunohistochemically.

Methods

Animal treatment

Two different dosage schedules were used. The treatment of group B is based on previous work in our laboratory [17, 18] and on preliminary experiments in our laboratory. For group A the same experimental design as previously described was used [19].

Group A. Male Sprague-Dawley rats (190 to 210 g) were s.c. injected twice a day (9:00 a.m.; 6:00 p.m.) with 10 mg/kg gentamicin during 10 days.

Group B. Female Wistar rats (225 to 250 g) received a daily dose of 400 mg/kg gentamicin (40 mg/ml) in three subcutaneous injections (8:00 a.m.; 4:00 p.m.; 12:00 p.m.) during two consecutive days. In addition, four experimental animals and four control animals did not receive the full treatment and were sacrificed after only one day treatment (further referred to as the day −1 sample), enabling the observation of early effects.

Control rats for the two groups received equal volumes of 0.9% NaCl under the same regimen. Animals had free access to rodent standard chow and tap water. One hour before sacrifice, 200 μCi ^{3}H-thymidine (NEN, Du Pont de Nemours, Wilmington, Delaware, USA) was injected intraperitoneally to evaluate cellular proliferation. The rats from group A were sacrificed by cervical fracture 24 hours after receiving their last injection. The rats from group B were killed between eight hours (that is, day −1 for those rats treated during one day, day 0 for the rats that completed the treatment of two days) and ten days after the last injection.

Sample collection

Serum samples were taken at the time of sacrifice. After exposure of the abdominal cavity, kidneys were decapsulated and quickly removed. The right kidney and a part of the liver were snap-frozen in liquid nitrogen and stored at −80°C, until DNA- and RNA-extraction procedures were performed. The left kidney was cut into 1 mm thick transverse slices and processed for further histological analysis using different fixation procedures. A cortex sample from the left kidney was weighed and stored at −20°C for determination of the gentamicin content.

Serum creatinine

Creatinine values in the serum were measured in duplicate by a Jaffé-modified colorimetric reaction (Creatinine Merckotest, Diagnostica Merck, Darmstadt, Germany).

Gentamicin in the kidney cortex

Gentamicin was extracted from cortex samples as previously described [20]. The cortical accumulation of gentamicin [21] was measured with a commercial RIA-kit (Gammacoat [^{125}I]-Gentamicin Radioimmunoassay kit, Baxter Healthcare Corp., Cambridge, Massachusetts, USA).

Light microscopy and histoautoradiography

Tissue slices (1.5 mm thick) were immersed during four hours in Dubosq-Brazil fixative at room temperature, rinsed in 70% ethanol and embedded in low melting point paraffin (BDH Chemical Ltd., Poole, UK). Sections (4 μm) were cut and mounted on chrome(III)potassium-sulfate-gelatin coated microscope slides, rehydrated and covered with autoradiographic emulsion (Kodak NTB 2 from Technomara AG, Wallisellen, Germany). After exposure for two to seven weeks at 4°C, slides were developed. Before staining with periodic acid/Schiff reagent, silver grains were stabilized by gold latensification. Nuclei were counterstained with hematoxylin. Neighboring sections were stained with hematoxylin-eosine only.

Immunohistochemical stainings

Staining procedures were essentially as previously described [22]. For EGF, tissue slices (1.5 mm thick) were fixed on melting ice during 90 minutes in 4% formaldehyde (BDH Chemical Ltd.) buffered with 0.1 M Na-cacodylate pH 7.4 containing 1% $CaCl_2$. After washing, tissue was embedded in low melting point paraffin (BDH Chemical Ltd.). Sections (4 μm) were mounted on poly-L-lysine coated microscope slides and rehydrated. After equilibration in TSB (10 mM Tris-HCl pH 7.6; 0.9% NaCl; 0.1% Triton X-100 and 0.004% merthiolate) and incubation with normal goat serum (1/5) during 20 minutes, rabbit anti-mouse EGF antiserum (1/5000) (Amersham International plc., Amersham, UK) was applied for overnight incubation. After washing, sections were treated with biotinylated goat anti-mouse antiserum, followed by the addition of the avidine-biotine peroxidase complex (Vector Laboratories Inc., Burlingame, Vermont, USA). Peroxidase staining was performed with 3-amino-9-ethylcarbazole as a substrate. No staining was observed after applying antiserum that was previously incubated for 24 hours with a dilution series of mouse submaxillary gland EGF (Gibco Life Technologies, Inc., New York, New York, USA) from 18 ng/ml to 18 μg/ml.

Tissue slices for leukocyte stainings were fixed the same as for EGF staining. Sections (4 μm) were mounted on poly-L-lysine coated microscope slides and treated for five minutes with 0.003% trypsin III (Sigma Chemical Co., St. Louis, Missouri, USA) in 10 mM Tris-HCl buffer pH 7.3. After washing in TSB and treatment with normal horse serum (1/5), the sections were incubated overnight with the primary antibodies OX1 (1/6000), OX8 (1/4000) or OX41 (1/1600) (Serotec, Oxford, UK). The OX1 Mab recognizes the rat leukocyte common antigen, which is present on all marrow-derived leukocytes, that is, thymocytes, bone marrow cells, peripheral lymphocytes and macrophages [23]. OX8 reacts with T cytotoxic/suppressor cells ($CD8^+$) and natural killer cells [24]. OX41 is specific for macrophages, polymorphic nuclear cells and dendritic cells, expressing the $CD11^+$ antigen [25]. The appropriate dilutions were determined in preliminary experiments. Endogeneous peroxidase activity was destroyed by immersion in methanol, followed by 30 minutes in 0.03% hydrogen peroxide. After washing, biotinylated horse anti-mouse serum was added for 30 minutes. Further staining was the same as for EGF.

Leukocyte cells were quantified in six microscopic fields for each animal by means of an image analyzer system (Vicom

Systems Inc., USA), and positive areas were expressed as a percentage of each randomly selected field.

^{3}H-thymidine incorporation

Genomic DNA was isolated from the lower part of the right kidney and from the liver, the latter to check the organ specificity of cell proliferation. Briefly, tissue was homogenized in 150 mM NaCl/100 mM EDTA pH 8.0 in a Dounce homogenizer. Cells were lysed by the addition of 0.2 volumes 10% SDS and $NaClO_4$ to a final concentration of 1 M. Proteins were phenol/chloroform-extracted and the genomic DNA was ethanol-precipitated. The resulting DNA slurry was dissolved in 10 mM Tris-HCl/1 mM EDTA pH 8.0. Duplicate samples of the DNA extract were counted in Aqualuma scintillation fluid (Lumac bv., Schaesberg, The Netherlands) in a scintillation counter (Tri-Carb 2660 from N.V. Canberra Packard, Brussels, Belgium). Counting efficiency was estimated by an external standard in combination with the sample quenching curves. To correct the counting data for sample DNA content the concentration was determined by a colorimetric assay based on the diphenylamine reaction [26]. Serum- and acid-soluble tissue radioactivity was counted to assure consistent intraperitoneal absorption and tissue distribution of the radioactive label. Unreliable incorporation results (as indicated by the absence of a single labeled nucleus and the extreme low specific activity of the extracted DNA) from animals with an insufficient intraperitoneal absorption of the label (serum radioactivity less than 1250 dpm/μl) were excluded.

Morphological and histoautoradiographic analyses

Tissue injury was evaluated in the different renal tubular compartments (proximal tubules of cortex (S1-S2) and outer stripe of the outer medulla (S3), non-proximal tubules of cortex and outer stripe of the outer medulla) [27], based on following criteria: normal/undamaged, damaged cells, cellular necrosis, and completely necrotic. Damage included the presence of extensive vacuolization and the intracellular accumulation of PAS-positive inclusions. Even in regenerative conditions, distinction between proximal and non-proximal tubules could be made by the absence of a clearly recognizable intercellular delineation and eventually by the presence of a brush border in the proximal tubules. Non-proximal tubules were identified by the presence of a clearly recognizable intercellular delineation and by the presence of PAS-positive stripes perpendicularly orientated near the basal cell surface [28].

Labeled nuclei per mm^2 were counted in 20 microscopic fields in the cortex and the outer stripe of the outer medulla at a magnification of 312×, with a distinction made between proximal tubular cells, non-proximal tubular cells and interstitial cells.

Northern and slot blot analysis

Total RNA was extracted from the upper part of the right kidney using a guanidiniumthiocyanate procedure [29]. After one round of oligo-dT-cellulose chromatography [30], yield and purity of the poly-A+ fraction were determined spectrophotometrically. Probe specificity was assured by means of Northern blot analysis. The following cDNA inserts were used as a probe: a 3.3 kb Eco RI cDNA insert, containing the partial coding sequence of the rat prepro-EGF mRNA [31]; the 0.5 kb Eco RI cDNA insert of prigf1-1, containing the entire coding sequence and parts of the 3′ and 5′ untranslated region of the rat IGF-I mRNA [32]; the 2.3 kb Eco RI cDNA insert of prTGF$_{0,2}$, containing the partial coding sequence and part of the 5′ untranslated region of the rat preproTGF-α mRNA [33, 34]; the 1.0 kb Eco RI cDNA insert of pβas, containing the entire coding sequence of human TGF-β1 mRNA [35]; the 2.2 kb Eco RI cDNA insert of pc-*fos*(rat)-1, containing the entire coding sequence of rat c-*fos* mRNA [36]; the 1.9 kb Bam HI cDNA insert of pMVW-2, containing the entire coding sequence and parts of the 3′ and 5′ untranslated region of human PDGF-B [37] and the 0.3 kb Pst I cDNA insert of pSPβact72, containing part of the 3′ untranslated region of rat β-actin. Poly-A+ samples (5 μg) were fractionated electrophoretically through formaldehyde-1.2% agarose gels and fixed on positively charged nylon membranes (Hybond-N+ from Amersham International plc.) by alkaline vacuum transfer (Vacugene XL blotting unit from Pharmacia LKB Biotechnology, Uppsala, Sweden). For quantification purposes equal amounts of poly-A+ samples were slot blotted (Biodot SF microfiltration unit from Bio-Rad Laboratories, Richmond, Virginia, USA).

After amplification of the plasmids containing the inserts, cDNA probes were prepared according to standard procedures [38], and ^{32}P-labeled by random primed DNA synthesis [39] (Multiprime DNA labeling kit from Amersham International plc.) to a specific activity of 0.5 to 1.10^9 cpm/μg DNA. After overnight prehybridization at 42°C in 5× SSC, 0.1% SDS, 5× Denhardt's solution, 50 mM sodium phosphate pH 7.0, 0.25 mg/ml denatured sonicated salmon sperm DNA and 50% deionized formamide, the labeled probe was added to hybridize for 24 hours under the same conditions. Stringency of the washes for each probe were determined in preliminary experiments. Finally, blots were exposed to preflashed Fuji HRG autoradiographic film at −80°C in a film cassette with two intensifying screens. To quantify the growth factor response, hybridization signals of the slot blots were densitometrically scanned and integrated by a digital imaging system (Datacopy GS+ scanner and MacIntosh IIcx personal computer, Imagecopy and Scan Analysis software). Variations in isolation, application or transfer of the poly-A+ RNA were accounted for by reprobing the blots with a cDNA probe for rat β-actin. The expression of β-actin mRNA was inversely correlated with the amount of RNA eluting from the oligo-dT-cellulose columns (Spearmans rank correlation coefficient: −0.607; $P < 0.0001$), indicating that variation in β-actin expression was predominantly an artifact of a variable poly-A+ sample content after poly-A+ enrichment.

Statistics

Results are expressed as the mean ± SEM. Differences between the control group and the experimental groups were evaluated using the Mann Whitney U test. P values of 0.05 or less were considered as significant.

Results

Serum creatinine values

In group A, serum creatinine values were not elevated (data not shown). In group B the rise of the serum creatinine values, starting at day 0, gave evidence for an early decrease in

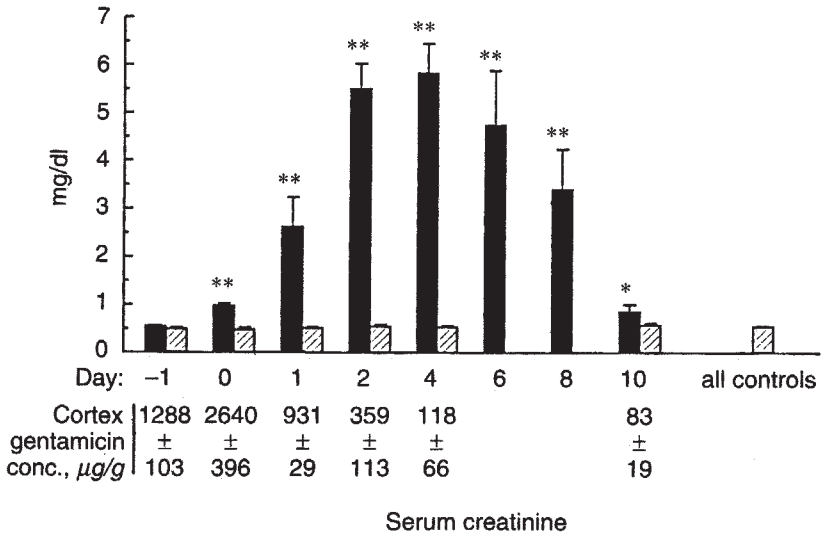

Fig. 1. *Serum creatinine values from group B.* Symbols are: (■) experimental; (▨) control. Each bar represents the mean ± SEM from four animals. * $P < 0.05$ versus all control rats ($N = 24$); ** $P < 0.01$ versus all control rats ($N = 24$).

glomerular filtration rate, whereas control rats maintained normal values throughout the experimental period (Fig. 1). Serum creatinine values from the treated rats peaked at day 4 (5.8 ± 0.6 mg/dl) and fell to nearly normal values at day 10.

Gentamicin accumulation in kidney cortex

After two days of treatment gentamicin in the kidney cortex of group B had reached a maximal concentration and decreased quickly thereafter (Fig. 1).

Morphological studies on tissue injury

By light microscopy, no clear morphological damage was observed in group A, except for the presence of some PAS-positive inclusions in proximal convoluted tubular cells (data not shown).

It clearly appeared from our morphological data from group B that even at high doses the segment specificity of the nephrotoxic action of gentamicin was preserved (Fig. 2). In the cortex the majority of the proximal tubular cells (S1-S2) were injured at day 0. In the next days, most of the proximal tubules became completely necrotic. The denuded basement membrane became gradually covered with flattened, labeled epithelial cells at day 6 (Fig. 3), and by day 10 tubular morphology was restored in most animals. By contrast, minor lesions were noticed in the other tubular segments: necrotic proximal tubules in the outer stripe of the outer medulla (S3) were exceptional, injuries being mostly confined to shedding of the brush border and the accumulation of PAS-positive granules in the cytoplasm. Although some disrupted cells were present in the non-proximal tubules from both cortex and outer stripe of the outer medulla, no necrotic areas were observed.

Proliferative response

In group B the renal proliferative response started at day 0 (Fig. 4A). Maximal proliferation occurred at day 6. The incorporation in liver DNA was not significantly elevated at any time (Fig. 4B).

Labeling indices for individual cell types were calculated from histoautoradiographical data. Cellular proliferation in group B reflected the incorporation data of the renal DNA extract. In the histological compartments essentially three proliferative waves were observed (Fig. 5). First, the number of labeled interstitial cells from cortex and outer stripe of the outer medulla rose very early (day 0). At day 6, interstitial proliferation faded away but at day 10 proliferative activity was still present. Second, proliferation in non-proximal tubular cells and in the proximal tubular cells from the outer stripe of the outer medulla started at day 2, and decreased slowly towards day 10, not yet being normalized at that time. Third, the major proliferative wave, localized in the proximal tubular cells from the cortex (S1-S2), started only at day 4 and peaked at day 6.

Glomerular cell proliferation was not significantly elevated (data not shown).

Interstitial infiltration

Since a profound interstitial proliferation was present in group B, the identity of the interstitial infiltrates was investigated immunohistochemically. In normal interstitium, 0.3% of the investigated area was OX1-positive (Fig. 6). In the experimental rats, a massive leukocyte infiltration was present throughout the observation period, being maximal at day 2 (12.7%). T suppressor/cytotoxic cells, as revealed by the OX8 Mab, covered 0.2% of the investigated area in control rats. In the gentamicin-treated animals, the OX8-positive cells infiltrated from day 0 onward. While OX41-positive cells (macrophages/granulocytes) were occasionally observed in control rats (0.02%), a transient infiltration was prominent in the experimental rats, peaking at day 4 (1.6%).

Growth factor expression

By means of Northern blot hybridization, the mRNA expression of several polypeptide growth factors was investigated in the kidney (Fig. 7). An intense 5.0 kb band revealed the abundant presence of the preproEGF transcript in the kidney, as has been reported by others [40–43]. For IGF-I, three distinct bands of 7.0, 1.6 and 1.1 to 0.6 kb were observed. This profile is in accordance with previous results [32] and since IGF-I is encoded by a single copy gene probably the consequence of

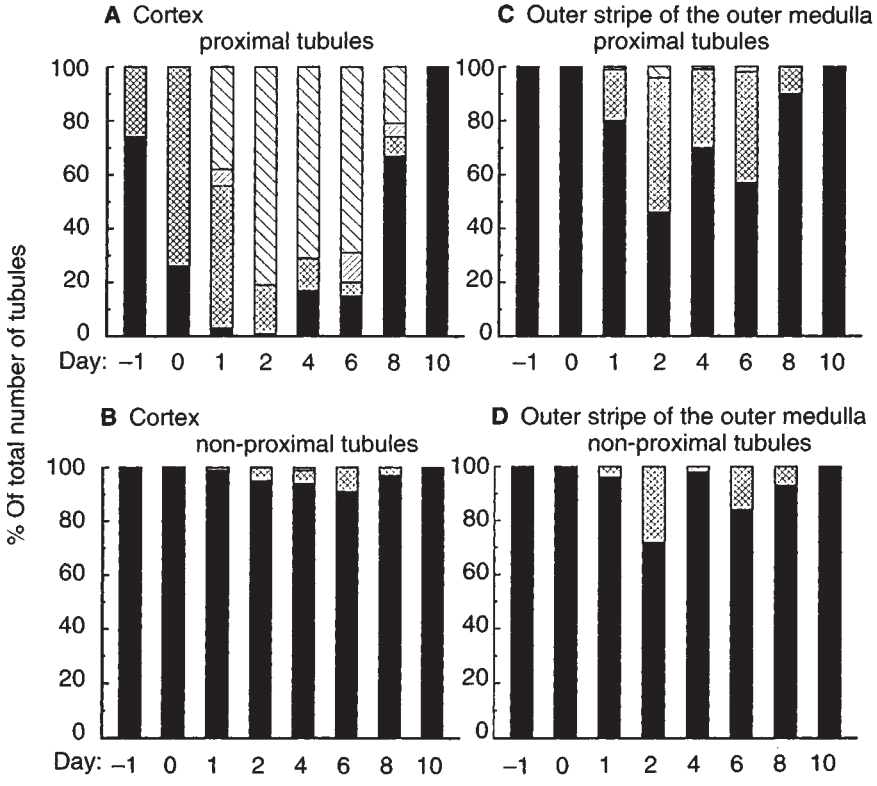

Fig. 2. *Morphometric analysis of the injury in different renal tubular compartments from group B.* Symbols are: (▧) completely necrotic; (▨) cellular necrosis; (▩) damaged cells; (■) normal/undamaged cells.

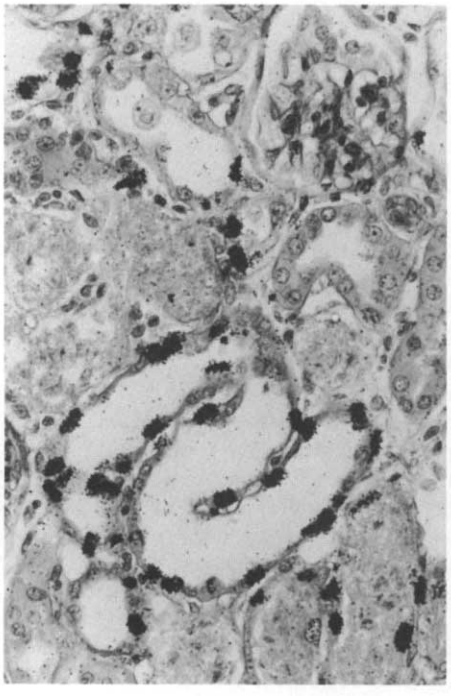

Fig. 3. *Photomicrograph of proliferating renal cells (magnification × 180) in an animal from group B six days after the treatment.*

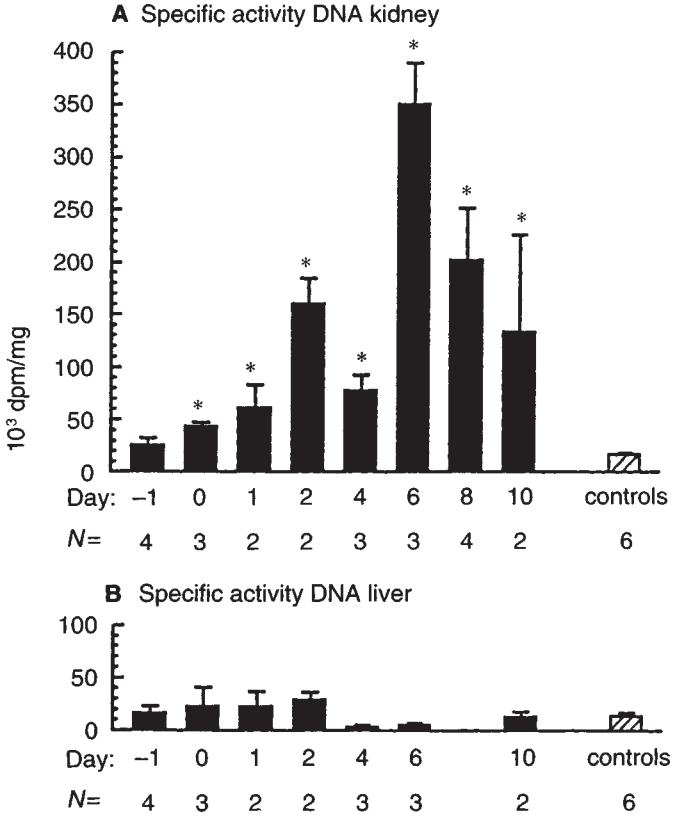

Fig. 4. *DNA specific activity from group B.* Each bar represents the mean ± SEM. * $P < 0.05$ versus the group of six control rats (three each from days −1 and 10).

alternative splicing of the IGF-I mRNA [44, 45]. A very weak band of 4.8 kb was detected using the rat TGF-α probe. The signal represents the full-length transcript [33, 34, 46]. The

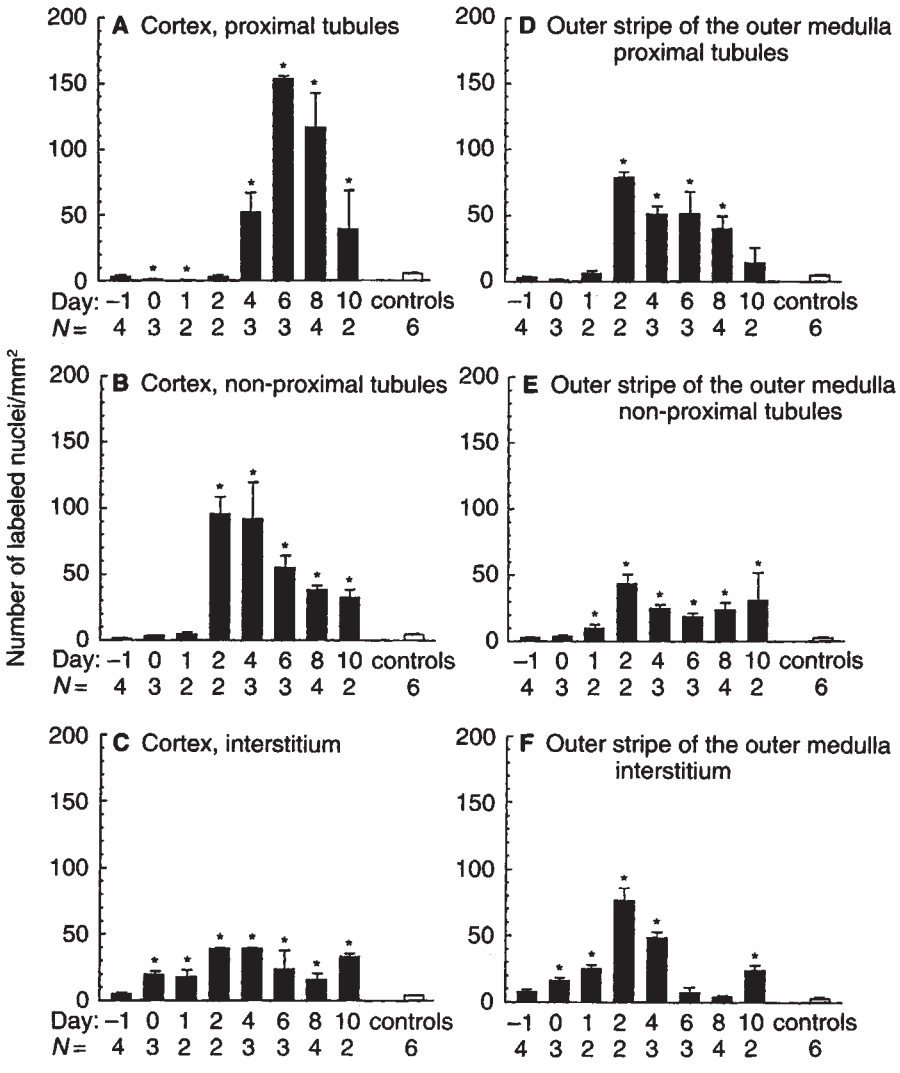

Fig. 5. *Labeling index in different renal compartments from group B.* Each bar represents the mean ± SEM. * $P < 0.05$ versus the group of six control rats (three each from days −1 and 10).

human TGF-β1 probe yielded a 2.5 kb band of moderate intensity, which matches the transcript of the rat's equivalent [47–50]. The presence of the PDGF-B in the kidney was confirmed by the presence of a single 3.8 kb transcript [37, 51, 52]. Finally, the presence of the 2.1 kb c-*fos* transcript in the normal rat kidney was very low, which is in agreement with previous reports [36, 53].

In group B, EGF mRNA fell to nearly undetectable levels at day 1 and remained low thereafter (Fig. 8). However, the level of the transcript was increasing again at the end of the observation period (day 10) as compared to day 8 ($P < 0.05$). Analogous to EGF, IGF-I mRNA level was also decreased, although not as dramatically as for EGF and, by contrast, it was completely normalized at day 10. The PDGF-B transcript increased significantly towards the end of the observation period. No consistent changes were observed in the level of the other transcripts examined, that is, TGF-α, TGF-β1 and c-*fos*. All values were standardized against β-actin mRNA expression.

EGF immunoreactivity

Immunohistochemical staining revealed that EGF expression in normal kidney was confined to the thick ascending limb of Henle (TAL) and the distal convoluted tubule (DCT) (Fig. 9). Staining was most prominent at the apical cell surface. No staining for EGF was observed in the macula densa. In group B, immunoreactive EGF decreased after 1 day of gentamicin administration, and did not reappear until days 8 to 10, in analogy to the disappearance of the EGF mRNA. However, the initial loss was confined to the distal tubules from cortex, that is, the area in which the severe proximal tubular necrosis was localized. Thereafter, EGF expression was also lost in the distal tubular cells of the outer stripe of the outer medulla. EGF staining started to reappear at day 10 similar to the Northern results. Immunoreactive EGF was never observed in proximal tubular cells. In group A neither a loss of EGF immunoreactivity nor its appearance in proximal tubular cells were observed.

Discussion

The possible involvement of several growth factors in the triggering and maintenance of the observed proliferation events was investigated at the mRNA level. A strong decrease in the expression of EGF was observed during the injury and the recovery phase in group B, but not in group A, both at the messenger and the protein levels. This illustrates that substantial injury is a prerequisite to affect the expression levels of

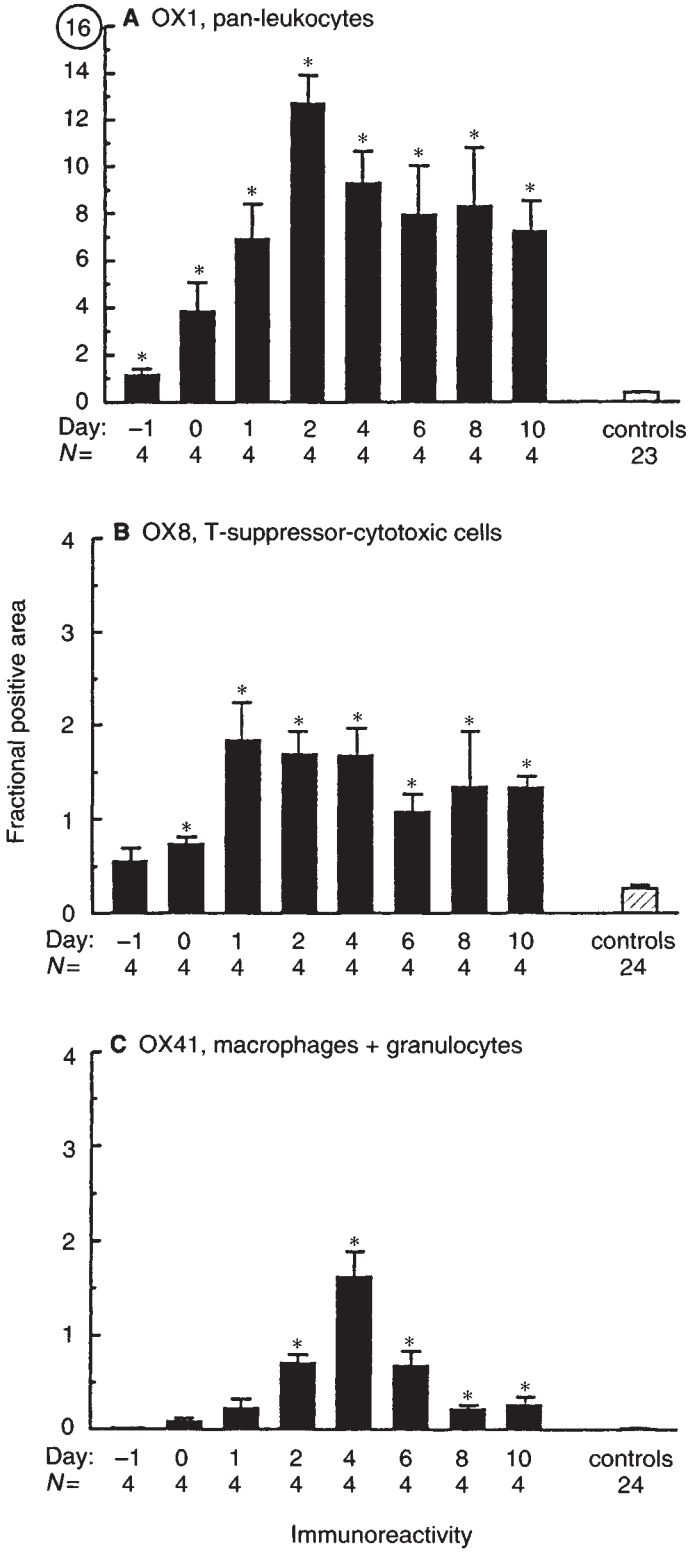

Fig. 6. *Positive fractional area of the immunoreactive infiltrating cells.* Each bar represents the mean ± SEM. * $P < 0.05$ versus the group of 24 control rats.

EGF, and of IGF-I. A similar loss of EGF mRNA expression, together with a decline in urinary EGF output, has been observed in other models of acute renal failure, that is, after ischemia and cisplatin administration [40, 41]. A direct interaction with gentamicin as the cause of this decline is therefore unlikely. Whether the concomittant decrease of EGF and its messenger has to be ascribed to a decline in transcription rate and/or a decreased mRNA stability remains to be determined. It is also not clear what causes the topographical association between the initial disappearance of EGF from the distal tubular segments and the adjacent site of proximal tubular injury. This phenomenon was also observed in preliminary experiments using mercurychloride as an inducer of segment-specific toxic lesions in the proximal straight tubules of the outer stripe of the outer medulla (unpublished observations).

The question remains whether the decreased EGF is the result of damage to the distal tubular cells, whether it is due to functional alterations in the distal nephron, or whether it is part of a specific control mechanism.

The first possibility is unlikely since clear distal tubular necrosis was absent; the small number of necrotic cells in the distal tubules that possibly escaped our observations cannot explain the virtual disappearance of EGF from the entire length of the distal tubule. Furthermore, no morphological damage had occurred to this part of the nephron when EGF started to disappear.

As for the possible role of a local decrease in EGF concentration acting as a specific regulatory signal during kidney regeneration, there are a number of indications which support this hypothesis. EGF can transiently arrest A431 human epidermoid carcinoma cells (that bear EGF receptors abundantly) in their G2-phase [54–56]. Furthermore, EGF seems to inhibit organ growth in neonatal rats [57, 58]. It is noteworthy in this regard that EGF mRNA cannot be detected in neonatal mice until four days after gestation [59], nor can it in human fetal tissues [60]. Also, EGF mRNA is decreased in congenital polycystic kidneys in mice [42, 61]. Nevertheless, the number of reports in this context are few in comparison to the number of descriptions of EGF as a stimulator of mitosis [62–67].

On the contrary, several facts make it difficult to understand how such a mitogenic activity could be exerted during proximal tubular regeneration. We could not demonstrate an autocrine role for renal EGF, being unable to detect any EGF immunoreactivity in proximal tubular cells, neither after mild, nor after severe proximal tubular injury. Perhaps, as Laurent and co-workers suggest, the redistribution of EGF immunoreactivity which they have observed using an immunogold technique [14, 15], indicates an increased binding of EGF to its receptor. However, this needs confirmation since it is still not clear how the renal EGF, localized at the luminal plasma membrane of distal tubular cells could bind to the EGF receptors of the proximal tubular cells that are exclusively localized at their basolateral membranes [68]. Although transcytosis of EGF in cultured renal epithelial cells has been described, it was directed from the basolateral towards the luminal surface [69]. Despite the fact that no EGF receptor analysis was performed in the present study, the relevance of disruption of the tight junctions and loss of tubular cell polarity, which would allow distal tubular EGF to reach its receptor, could be questioned, since distal tubular EGF is virtually absent in our model when its mitogenic properties would be most appropriate.

Nevertheless, other data indicate that EGF does have a beneficial effect on renal regeneration. Humes and co-workers

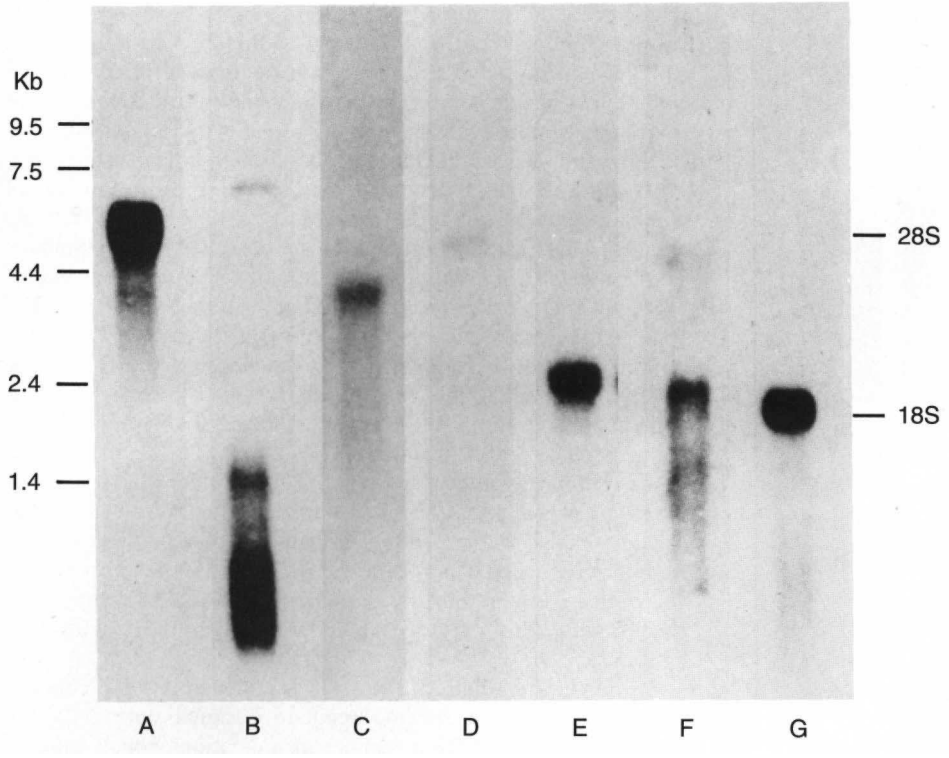

Fig. 7. *Northern hybridization of different mRNA transcripts detected in normal adult rat kidney, except for c-*fos *(group B after 2 days treatment).* **A.** EGF mRNA: one transcript of 5.0 kb. **B.** IGF-I mRNA: three transcripts of 7.0, 1.6 and 1.1 to 0.7 kb. **C.** PDGF-B mRNA: one transcript of 3.8 kb. **D.** TGF-α mRNA: one transcript of 4.8 kb. **E.** TGF-β1 mRNA: one transcript of 2.5 kb. **F.** c-*fos* mRNA: one transcript of 2.2 kb. **G.** β-actin mRNA: one transcript of 2.1 kb.

have reported a hastened recovery from ischemic and mercury chloride-induced acute renal failure after i.p. administration of EGF [70, 71]. Together with the higher EGF receptor density previously reported [72], this suggests that in these circumstances circulating rather than renal EGF could be important. On the other hand, also TGF-α, which is able to activate the EGF receptor [73], is shown to accelerate renal repair following ischemic injury [74]. However, the same enigma occurs as for renal EGF, since renal TGF-α has been localized in the collecting duct [75], that is, downstream from the site of injury. Furthermore, TGF-α mRNA remained relatively constant in our model.

Alternatively, EGF of renal origin might act at the lower urinary tract [43] in analogy to the activity of salivary gland EGF on the gastrointestinal epithelia [76], the more since it has been demonstrated that the renal EGF precursor can be cleaved by a membrane bound aprotinin-inhibitable proteinase [77, 78] and several kidney derived EGF-like peptides can be detected in the urine [79]. Furthermore, it is intriguing that the EGF precursor molecule contains several EGF-like domains in addition to the mature EGF [80, 81]. The homology with the LDL-receptor further suggests alternative functions, unrelated to cellular proliferation [82].

Several investigators have reported the collecting duct to be the major site of renal IGF-I expression [83–86]. The presently observed decrease in IGF-I mRNA content is not as dramatic as for EGF mRNA. Again, it is difficult to reconcile such a response with a role for renal IGF-I as a proliferation promoting substance involved in the regeneration of the kidney. Since there is evidence for the existence of an intrarenal EGF-IGF-I axis [87], the presently observed decline in preproIGF-I mRNA might be the result of a decreased paracrine stimulation of IGF-I mRNA production by the distal tubular EGF. However, this cannot explain the return to normal levels of IGF-I mRNA while renal EGF is still far below its normal level of expression.

In the present study, PDGF-B mRNA was the only growth factor that was significantly elevated. In addition to its role in the proliferation of fibroblasts and other cells of mesenchymal origin [88], PDGF elicits chemotactic responses in fibroblasts and smooth muscle cells and stimulates the deposition of collagen [89]. Some studies have suggested that PDGF and PDGF receptors may mediate destructive proliferative processes within tissue sites, including the kidney [90–92]. The tubulointerstitial fibrotic lesions that sometimes develop after severe tubular injuries [93] therefore may be foretold by the increased abundance of the PDGF-B transcript at the end of our experiment. Several studies have also demonstrated an induction of PDGF-B mRNA by TGF-β [94] and vice versa, TGF-β was induced in fibroblasts and infiltrating macrophages in PDGF-treated wounds [89]. The similar expression patterns of PDGF-B mRNA and TGF-β1 mRNA may be a further confirmation of this relationship, although TGF-β1 mRNA level was not significantly altered.

No consistent variation in the mRNA expression of TGF-α, TGF-β1 and c-*fos* was observed. However, this does not exclude a possible role for these factors in the regenerative events since posttranslational mechanisms such as receptor affinity changes, alterations in signal transduction or activation of latent forms are not always accompanied with a modified mRNA content. Also, since RNA extracted from the whole kidney was used, it was impossible to detect any local differences in expression that may have occurred for these factors. The broad interval in which the different renal celltypes reentered the cell cycle made it difficult to detect significant

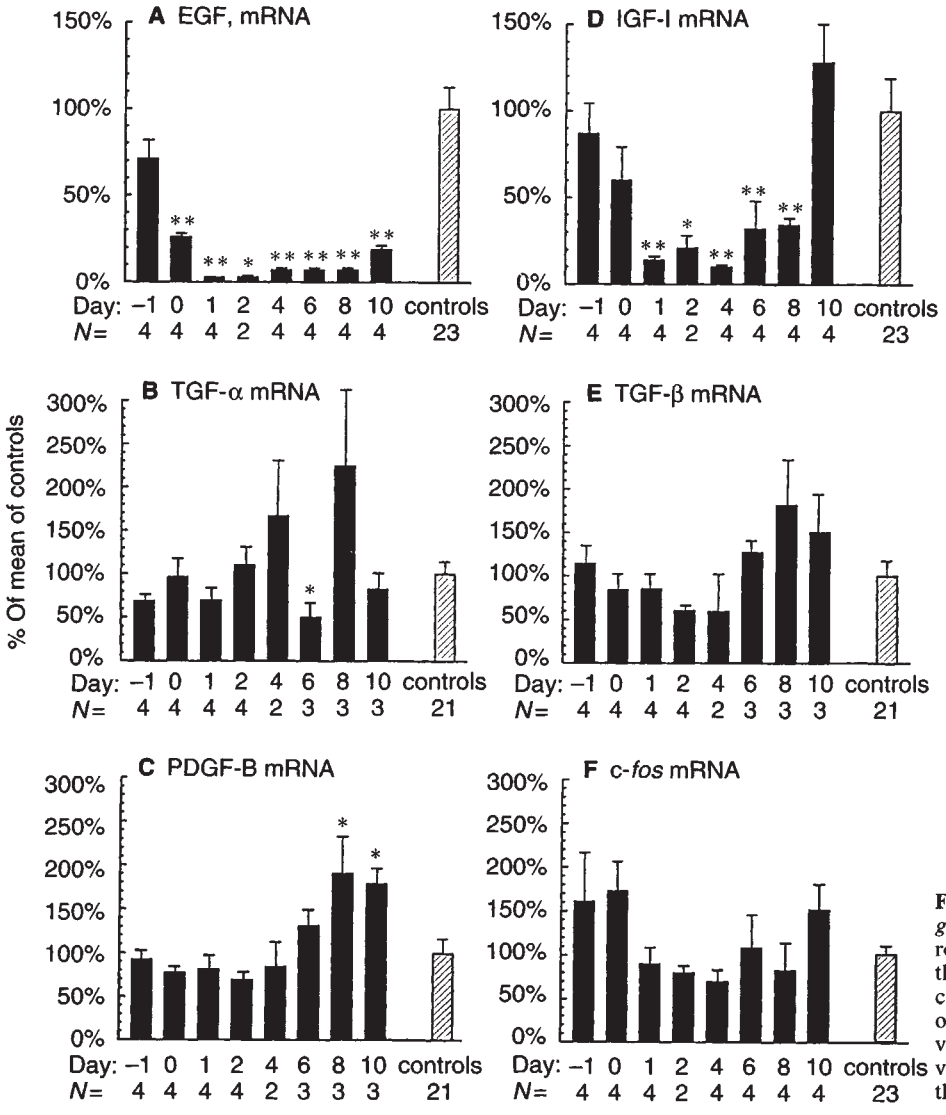

Fig. 8. *Slot blot densitometric analysis of the growth factor mRNA from group B.* Each bar represents the mean ± SEM as a percentage of the mean from the control animals. Values are corrected for variations in isolation, transfer, or application of the mRNA by normalization versus the β-actin mRNA content. * $P < 0.05$ versus the control group. ** $P < 0.01$ versus the control group.

variations for the c-*fos* mRNA, considering the very rapid and transient activation of the gene [95]. Furthermore, it has been suggested that c-*fos* induction in Swiss 3T3 cells is not obligatory for the mitogenic response for these cells [96].

The interstitial infiltration of leukocytes was massive, persistent and appeared before the onset of distinct tubular necrosis. Whether this observation plays a primary or a secondary role in renal injury is not known. However, mononuclear cells and macrophages can secrete soluble factors that can stimulate the resident fibroblasts in the renal interstitium [97]. The early infiltration may therefore eventually lead to interstitial fibrosis and progressive deterioration of renal function associated with chronic renal disease. The mechanism underlying the development of this cellular infiltrate is not clear. Since T cell infiltration was a prominent feature of this phenomenon, cell-mediated mechanisms may have contributed to it. Since renal tubular cells can produce proinflammatory cytokines such as TNFα, and can act as antigen presenting cells by expressing the MHC class II complex, it is possible that these cells have participated in the immune response [98]. Alternatively, the interstitial infiltration could be secondary to early cellular damage due to the lysosomal gentamicin overload [17, 21]. This attractive hypothesis unifies several models of tubulointerstitial injury, such as severe toxic and ischemic nephropathy, and proteinuria-induced tubulointerstitial nephritis, all characterized by tubular epithelial cell injury and disruption being the final common pathway [99–101]. The outcome largely depends on the severity and reversibility of the initial insult.

In summary, we have chronologically characterized a model in which gentamicin-induced toxic segment-specific tubular injury was followed by regeneration resulting in a complete recovery. In this model an early decreased preproEGF mRNA content, accompanied by the disappearance of renal EGF at the apical surface of the distal tubular cells, was seen simultaneously with the induction of distinct renal injury. Furthermore, distal tubular cells adjacent to the necrotic proximal tubules in the cortex have lost their EGF expression first, while immunoreactive EGF was never observed in proximal tubular cells. IGF-I mRNA decreased also but regained its normal expression after restoration of normal tissue morphology. The

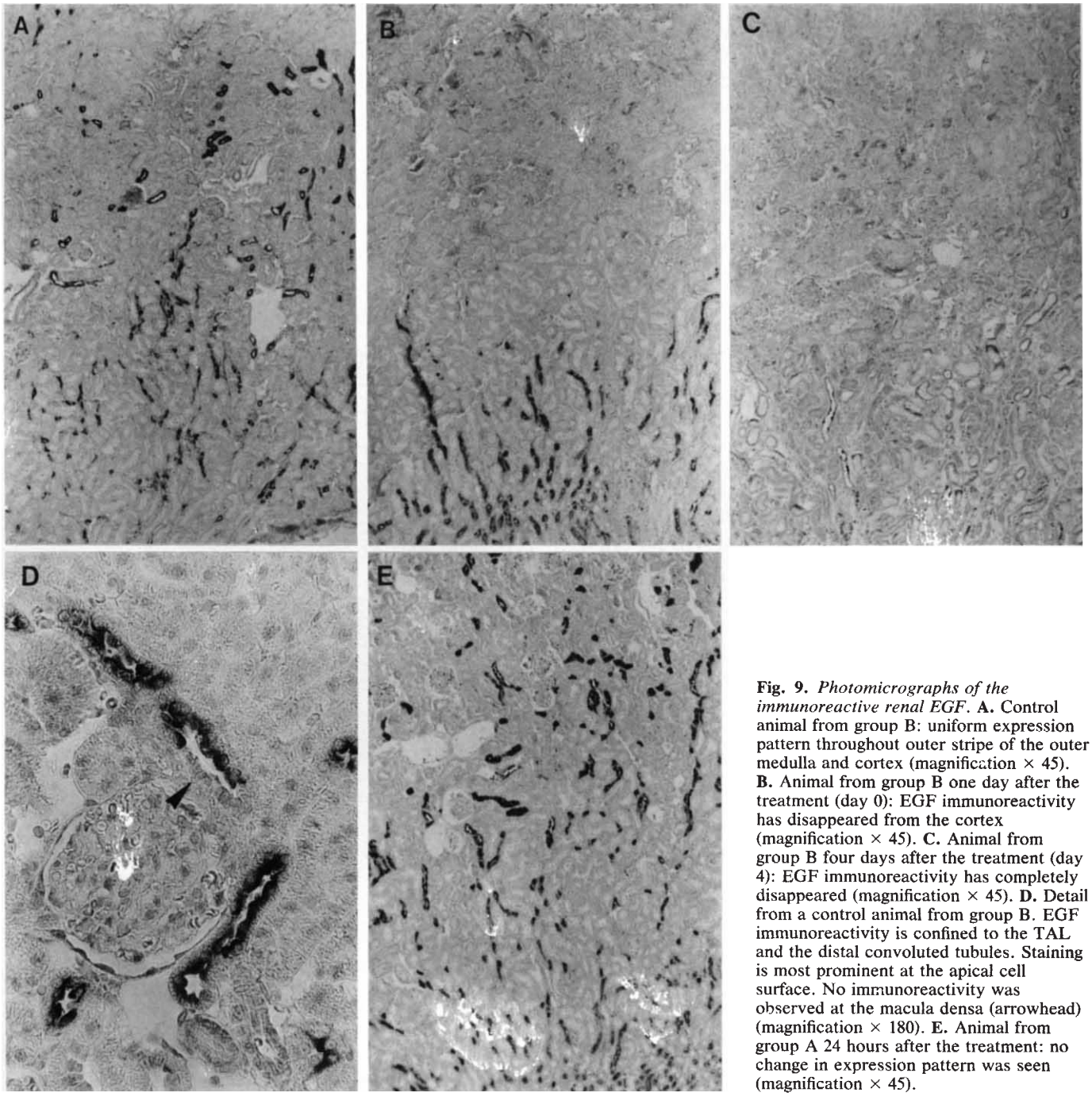

Fig. 9. *Photomicrographs of the immunoreactive renal EGF.* **A.** Control animal from group B: uniform expression pattern throughout outer stripe of the outer medulla and cortex (magnification × 45). **B.** Animal from group B one day after the treatment (day 0): EGF immunoreactivity has disappeared from the cortex (magnification × 45). **C.** Animal from group B four days after the treatment (day 4): EGF immunoreactivity has completely disappeared (magnification × 45). **D.** Detail from a control animal from group B. EGF immunoreactivity is confined to the TAL and the distal convoluted tubules. Staining is most prominent at the apical cell surface. No immunoreactivity was observed at the macula densa (arrowhead) (magnification × 180). **E.** Animal from group A 24 hours after the treatment: no change in expression pattern was seen (magnification × 45).

PDGF-B transcript was elevated significantly at the end of the observation period. The mRNA level of the other growth factors investigated did not change. No evidence for a participation of EGF or IGF-I of renal origin in the proliferative response after toxic proximal tubular necrosis was found so far.

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