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Increased epidermal growth factor in experimental diabetes related kidney growth in rats

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Summary Renal enlargement is a characteristic feature of human and experimental diabetes mellitus that may be predictive of subsequent nephropathy. In the streptozotocin diabetic rat kidney growth rapidly follows the induction of experimental diabetes but the mechanisms responsible for this growth are poorly understood. Epidermal growth factor (EGF) is a potent mitogen for renal tubular cells. Thirty one male Sprague-Dawley rats aged 13 weeks were randomised to receive either streptozotocin (diabetic, $n = 20$) or buffer (control, $n = 11$). Animals were studied on days 1, 3, 5 and 7 following streptozotocin. Diabetes was associated with a 3-fold increase in urinary EGF excretion (223 ± 15 vs 59 ± 5 ng/day, mean ± SEM, diabetic vs control, $p < 0.0001$) and 3–6 fold increase in renal EGF mRNA relative to controls ($p < 0.001$). A transient rise in kidney EGF protein was noted on day 1. There was no difference between diabetic and control animals with regard to intrarenal sites of EGF expression or in plasma EGF. These data suggest that the increased urinary EGF excretion in diabetic animals is the result of enhanced local production and that EGF is not stored for a prolonged period within renal tubular cells but is released following its synthesis. In the context of the known intrarenal actions of EGF this growth factor may play a role in the pathogenesis of diabetes related kidney growth. [Diabetologia (1997) 40: 778–785]

Keywords Diabetes mellitus, epidermal growth factor, kidney growth, mRNA.

Renal enlargement is a characteristic feature of human [1] and experimental diabetes mellitus [2]. In humans, renal enlargement may be predictive of subsequent nephropathy in patients with insulin-dependent [3] and non-insulin-dependent diabetes [4].

In the streptozotocin (STZ) rat model, increased kidney weight is detectable 36 h following the induction of diabetes and increases by approximately 20 % within 1 week [5]. The mechanisms responsible for this growth are poorly understood.

The mammalian kidney is a major site of synthesis of epidermal growth factor (EGF) [6]. EGF is thought to act in a paracrine, autocrine or juxtacrine manner [7] and not as a circulating hormone given its low plasma concentration [8]. In addition to its ability to promote cell growth and proliferation, EGF exerts a variety of effects on renal function [9].

Previous studies have demonstrated an increase in kidney EGF in the compensatory renal hypertrophy that follows uninephrectomy [10, 11]. An increase in urinary EGF has been demonstrated in early experimental diabetes [12] in the absence of a change in kidney EGF. Only one study has examined EGF gene expression in experimental diabetes and found no change in glomerular EGF mRNA [13] . Thus the site of origin of the augmented urinary EGF excretion in diabetes is not known although there is some

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Abbreviations: EGF, Epidermal growth factor; rRNA, ribosomal ribonucleic acid; STZ, streptozotocin; TGF-β, transforming growth factor-β; PDGF, platelet-derived growth factor; OD, optical density; AU, arbitrary units; ROD, relative optical density; DCT, distal convoluted tubule; TAL, thick ascending loop of Henle.

dispute regarding a possible glomerular source of EGF expression [9, 13].

The aim of the present study was to assess whether EGF gene transcription is altered in diabetes and to localise the sites of such change within the kidney.

Materials and methods

Animals. Thirty one male Sprague-Dawley rats aged 13 weeks were randomised to control and diabetic groups. Diabetes was induced in 20 rats by the intravenous administration of STZ 50 mg/kg body weight. The remaining 11 rats were injected with citrate buffer and served as controls. Diabetic rats were killed at 1 ($n = 4$), 3 ($n = 4$), 5 ($n = 4$) and 7 days ($n = 7$) following injection of STZ. All control animals were killed at 7 days following injection of vehicle. At each time point diabetic and control rats were used to study EGF gene expression and peptide content. Prior to decapitation animals were weighed, the kidneys were then excised, decapsulated and weighed. Kidneys were then snap frozen in liquid nitrogen and stored at –80 °C for later measurement of EGF mRNA (left kidney) and peptide content (right kidney). Blood, obtained at decapitation was collected in lithium heparin tubes, placed on ice, spun immediately, separated and stored at –20 °C. Plasma glucose at killing was measured by a glucose oxidase technique [14]. In addition, 4 diabetic and 4 control rats were housed in metabolic cages in order to obtain daily urine collections. At 7 days these animals were killed and kidneys were fixed in 10 % neutral buffered formalin for in situ hybridisation studies (left kidney) and paraformaldehyde for immunohistochemistry (right kidney). All aspects of the experiment were approved by the animal ethics committee of the Austin and Repatriation Medical Centre.

RNA preparation and analysis. Tissues were homogenised (Ultra-Turrax; Janke and Kunel, Staufen, Germany) and total RNA was isolated by the acid guanidinium thiocyanate-phenol-chloroform extraction method [15]. RNA purity and concentration were determined spectrophotometrically. Twenty microgram samples were denatured and electrophoresed through 0.8 % agarose formaldehyde gels. RNA integrity was verified by examination of the 28S and 18S ribosomal bands of ethidium bromide stained material under ultraviolet light. RNA was then transferred onto nylon filters (Hybond-N; Amersham, Amersham, UK) by capillary action and fixed by ultraviolet irradiation. Filters were probed with a 400 base pair Eco RI/Hind III cDNA coding for murine EGF (gift of Dr. P. Fuller, Monash Medical Centre, Clayton, Australia) previously used in studies of rat kidney [16] reflecting the high degree of sequence homology between rat and mouse EGF mRNA [17]. The probe was labelled with [α-$^{-32}$P] dCTP by random primed DNA synthesis (Boehringer Mannheim, Mannheim, Germany). Hybridisation was performed at 42 °C for 24 h in 50 % formamide, 45 mmol/l Na_2HPO_4, 5 × Denhardt's solution, 0.5 % SDS and sonicated salmon sperm DNA. Filters were then washed in solutions of decreasing ionic strength and increasing temperature. The final stringency was 0.1 × standard saline citrate, 0.1 % SDS for 20 min at 42 °C. Filters were then exposed to X-ray film (Kodak X-Omat; Eastman-Kodak, Rochester, N. Y., USA) at –80 °C for 1–5 days. The relative intensity of autoradiograms was determined by scanning densitometry (LKB Ultroscan XL; Bromma, Sweden). All results were corrected for differences in RNA loading by rehybridisation with an oligonucleotide probe for 18S rRNA end labelled with [α-$^{-32}$P] dCTP by terminal transferase (Boehringer Mannheim).

In situ hybridisation. An anti-sense riboprobe was generated as previously described. In brief, the cDNA coding for EGF used in Northern analysis was cloned into pGEM 3Z (Promega, Maddison, Wis., USA) and linearized with Hind III. An antisense riboprobe was then generated using T7 RNA polymerase. Purified riboprobe length was adjusted to approximately 150 bases by alkaline hydrolysis. Four micrometer thick sections were cut onto slides precoated with 3-aminopropyltriethoxysilane and baked overnight at 37 °C. Tissue sections were dewaxed and rehydrated in graded ethanol and milliQ water, equilibrated in P buffer (50 mmol/l Tris-HCl, pH 7.5, 5 mmol/l EDTA) and incubated in 125 μg/ml Pronase E in P buffer for 10 min at 37 °C. Sections were then washed in 0.1 mol/l sodium phosphate buffer, pH 7.2, rinsed in milliQ water, dehydrated in 70 % ethanol and air dried. Hybridisation buffer containing 2×10^4 cpm/μl riboprobe in 300 mmol/l NaCl, 10 mmol/l Tris-HCl, pH 7.5, 10 mmol/l Na_2HPO_4, 5 mmol/l EDTA, pH 8.0, 1 × Denhardt's solution, 50 % formamide, 17 mg/ml yeast RNA, 10 % weight/volume dextran sulfate, was heated to 85 °C for 5 min. Twenty-five μl of this solution was then added to each section. Hybridisation was performed overnight at 60 °C in 50 % formamide humidified chambers. As controls for non-specific signal sections were incubated with sense riboprobe or treated with RNAse prior to hybridisation. Slides were washed in 2 × SSC containing 50 % formamide prewarmed to 50 °C to remove coverslips. Sections were then washed in the above solution for 1 hour at 55 °C, rinsed 3 more times in RNAse buffer (10 mmol/l Tris-HCl, pH 7.5, 1 mmol/l EDTA, pH 8.0, 0.5 mol/l NaCl) and then incubated with RNAse A (150 μg/ml) for 1 h at 37 °C. Sections were later washed in 2 × SSC for 45 min at 55 °C, dehydrated in graded ethanol, air dried and exposed to Kodak X-Omat autoradiographic film for 3 days. Slides were then dipped in Ilford K5 nuclear emulsion (Ilford, Mobberley, Cheshire, UK), stored in a light-free box with desiccant at 4 °C for 21 days, immersed in Kodak D19 developer, fixed in Ilford Hypam and stained with haematoxylin and eosin.

Quantitative autoradiography. The relative distribution of EGF gene expression within individual kidneys was quantified by comparing EGF mRNA in the cortex with that in the outer medulla. These regions were selected to reflect the anatomical distribution of the major sites of EGF expression: the distal convoluted tubule (DCT) and thick ascending limb of the loop of Henle (TAL) with the former located in the cortex and the latter found predominantly in the outer medulla [19]. Densitometry of autoradiographic images obtained by in situ hybridization was performed by computer-assisted image analysis as described by Baskin and Stahl [20] using the MCID system (Image Research, Ontario, Canada). In brief, in situ autoradiographic images were placed on a uniformly illuminating fluorescent light box (Northern Light Precision Luminator Model C60; Image Research, Ontario, Canada) and captured using a video camera (Sony Video Camera Module CCD, Tokyo, Japan) connected to an IBM AT computer with a 512 × 512 pixel array imaging board with 256 gray levels. Following appropriate calibration by constructing a calibration curve of optical density versus radioactivity [21, 22] quantitation of digitalized autoradiographic images was performed with the MCID software and expressed as relative optical density (ROD). Cortex:outer medulla ROD was analysed in control and day 7 diabetic animals.

EGF peptide. Renal homogenates were prepared in 2.5 ml of ice cold normal saline using a glass and teflon homogeniser.

Table 1. Body and kidney weight, plasma and kidney EGF in experimental animals

	Control	Diabetes			
		Day 1	Day 3	Day 5	Day 7
n	11	4	4	4	7
Glucose (mmol/l)	7.2 ± 0.5	18 ± 0.8[c]	21 ± 3.0[c]	20 ± 1.0[c]	20 ± 0.7[c]
Rat weight (g)	373 ± 9	364 ± 18	352 ± 11	299 ± 20[b]	311 ± 17[b]
Left kidney weight (g)	1.1 ± 0.4	1.1 ± 0.6	1.2 ± 0.2	1.3 ± 0.4[b]	1.3 ± 0.1[b]
Renal EGF ng/g	7.3 ± 0.7	11.7 ± 1.5[a]	8.6 ± 1.2	5.7 ± 0.3	8.2 ± 1.0
Plasma EGF ng/ml	1.6 ± 0.3	1.7 ± 0.1	1.6 ± 0.1	1.5 ± 0.2	1.7 ± 0.2

Results are expressed as mean ± SEM. [a] $p < 0.05$, [b] $p < 0.01$, [c] $p < 0.001$

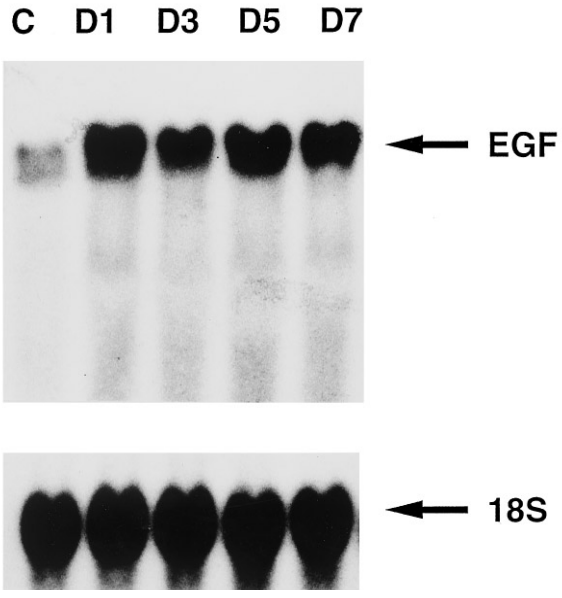

Fig. 1. Expression of kidney EGF mRNA following induction of STZ diabetes. Representative Northern blot of EGF in control (C) and rats studied at 1, 3, 5 and 7 days (D1, D3, D5 and D7) duration of diabetes. Expression of 18S rRNA is also shown

Samples were centrifuged at 10 000 *g* for 20 min at 4 °C. The supernatant was decanted and stored at –20 °C for later assay of EGF. Urine, plasma and renal homogenate were assayed for EGF by radioimmunoassay using a kit for mouse EGF and a rat EGF standard (Amersham). The sensitivity of the assay was 4 ng/μl and the intra-assay coefficient of variation was 6.5 %.

EGF immunohistochemistry. Five μm sections were rehydrated and treated with 1 % H_2O_2/methanol followed by incubation in Protein Blocking Agent (Lipshaw-Immunon, Pittsburgh, Penn., USA) for 20 min at room temperature. Sections were then incubated with anti-EGF antibody (Serotec, Oxford, UK) for 30 min at room temperature, washed in phosphate buffered saline and incubated with biotinylated goat anti-rabbit immunoglobulin (DAKO, Carpinteria, Calif., USA) and peroxidase-conjugated strepavidin (DAKO). Peroxidase conjugates were subsequently localized using diaminobenzidine tetrahydrochloride (DAB) as a chromogen. As a control for anti-EGF immunoreactivity, EGF peptide (Serotec) was preincubated with anti-EGF antibody and used in place of the EGF antibody alone in the normal staining procedure. Tissues treated in this manner showed no positive staining. Sections were examined by two independent observers blinded to the disease status of the animal.

Statistical analysis. Data are expressed as mean ± SD unless stated otherwise. Statistical significance of Northern blot analyses, quantification of in situ hybridisation autoradiographs and tissue and plasma EGF peptide were determined by ANOVA for multiple comparisons. Results obtained for serial urinary EGF were analysed by ANOVA for repeated measures. Analyses were performed using the Statview II + Graphics (Abacus Concepts, Berkeley, Calif., USA) package on an Apple Macintosh Centris 650 (Apple Computer Inc, Cupertino, Calif., USA) computer. A p value less than 0.05 was considered statistically significant.

Results

Rats that had received STZ were all diabetic (Table 1). Diabetes was associated with reduced body mass and increased kidney weight when compared with control animals (Table 1).

Northern analysis. Compared with control animals, kidney EGF mRNA in diabetic rats was increased 3–4 fold on day 1 with further overexpression continuing throughout the study period ($p < 0.001$) (Figs. 1, 2). In the diabetic group there was no relationship between EGF expression (mRNA or urinary EGF protein) and either body weight or plasma glucose.

In situ hybridisation and immunohistochemistry In situ hybridisation and immunohistochemistry demonstrated EGF mRNA and protein in the DCT and TAL. No change in the pattern of distribution of either mRNA or protein was found in kidneys from diabetic rats compared with control animals (Figs. 3–6) with no sites of aberrant EGF expression in diabetic rats. Specificity of immunohistochemical staining and in situ hybridisation for EGF was confirmed by the absence of labelling in negative controls (Figs. 7 and 8). Examination by blinded observers did not detect a difference in the relative distribution of EGF protein or mRNA in the DCT compared with TAL in control compared to diabetic animals. Similarly, quantitative autoradiography showed no difference in the cortex:outer medulla ROD between control and diabetic rats (0.36 ± 0.01 vs 0.40 ± 0.08, control vs diabetic).

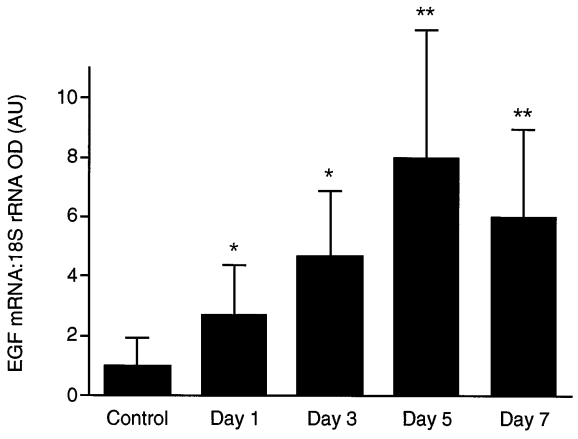

Fig. 2. Quantitation of kidney EGF mRNA in control and diabetic animals. Data are means ± SD of the ratio of optical density (OD) in arbitrary units (AU) for EGF mRNA to that of 18S rRNA. * $p < 0.05$, ** $p < 0.01$

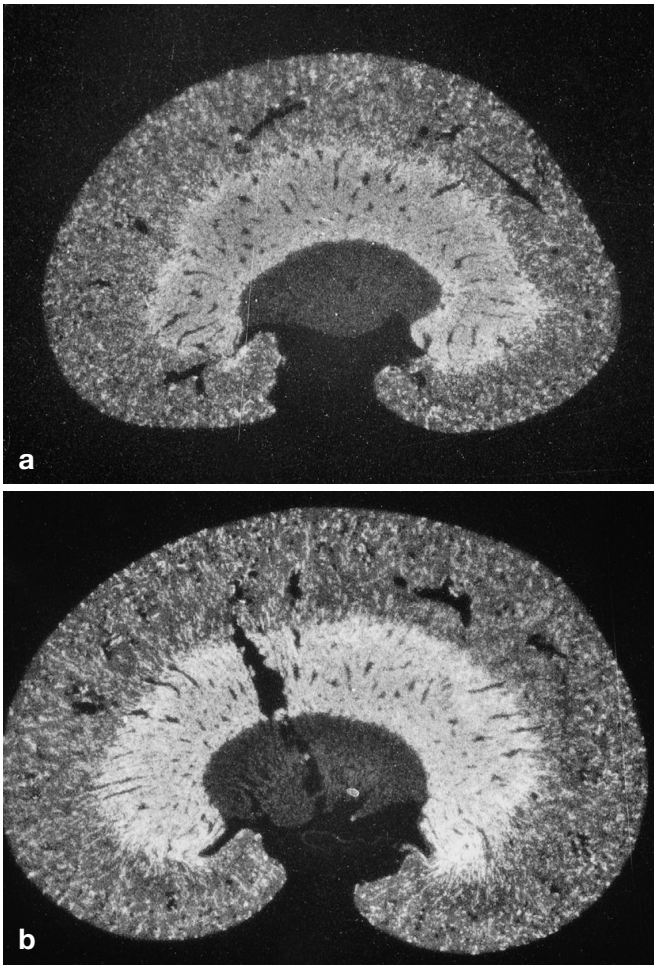

Fig. 3. In situ hybridisation of EGF mRNA in sagittal section of kidney from control (**a**) and diabetic (**b**) rat demonstrating hybridisation in the cortex and outer medulla

EGF peptide. Urinary EGF increased on day 1 in diabetic animals, then increased further achieving a plateau level 3 times greater than control animals and baseline levels ($p < 0.0001$, Fig. 6). Kidney EGF rose transiently on day 1 and then returned to levels similar to those of control animals (Table 1). There was no significant difference between control and diabetic groups with regard to plasma EGF at any time point (Table 1).

Discussion

The present study demonstrates that kidney EGF gene expression and peptide excretion increase rapidly following induction of experimental diabetes. The increase in renal EGF mRNA in the diabetic group and absence of a difference in plasma EGF between groups suggest that the increase in urinary EGF excretion in diabetic animals is the result of local production rather than altered plasma clearance. The transient and relatively small increase in kidney EGF in diabetic animals is consistent with the view that EGF is not stored within renal tubular cells for a prolonged period but that following its production the precursor molecule undergoes proteolytic cleavage releasing mature EGF. Furthermore, sites of EGF mRNA were not different in control and diabetic animals suggesting that induction of EGF transcription at aberrant sites within the kidney did not account for EGF overexpression found in the present study.

Diabetes is associated with profound pathophysiological changes in the kidney some of which may be a consequence of the demonstrated increase in EGF. Firstly, experimental diabetes is associated with a rapid increase in kidney size despite a loss of body weight. The factors that mediate this growth are poorly understood. Early increases in renal IGF-I have been demonstrated in experimental diabetes [24, 25]. These findings are consistent with the ability of EGF to increase renal IGF-I production in a dose dependent manner [26] and suggest that these two growth factors acting in concert may be responsible for renal growth in diabetes. In vitro, EGF is a mitogen for tubular epithelial and possibly other renal cell types [27]. For instance, the renal growth that follows unilateral nephrectomy is associated with increased renal EGF [11, 10] and the associated tubular cell hyperplasia can be reduced by the administration of anti-EGF antibodies [28]. In acute tubular necrosis following ischaemic injury [29, 30] or mercuric chloride toxicity exogenously administered EGF accelerates tubular regeneration and recovery. The factors responsible for increased EGF during early diabetes remain speculative. Proto-oncogenes which modulate gene transcription such as *c-fos* are increased following glucose exposure *in vitro* and within 24 h of the

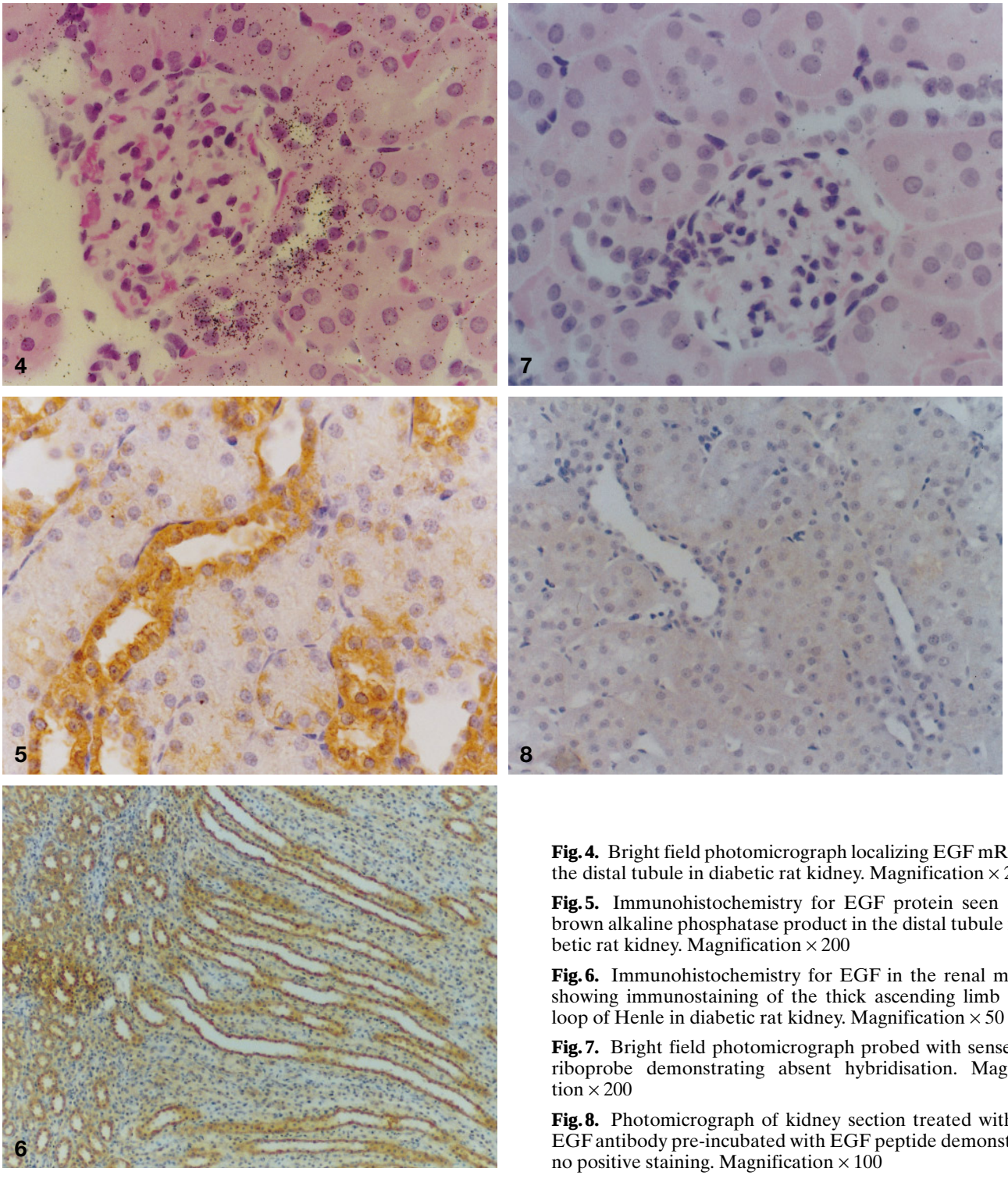

Fig. 4. Bright field photomicrograph localizing EGF mRNA to the distal tubule in diabetic rat kidney. Magnification × 200

Fig. 5. Immunohistochemistry for EGF protein seen as the brown alkaline phosphatase product in the distal tubule in diabetic rat kidney. Magnification × 200

Fig. 6. Immunohistochemistry for EGF in the renal medulla showing immunostaining of the thick ascending limb of the loop of Henle in diabetic rat kidney. Magnification × 50

Fig. 7. Bright field photomicrograph probed with sense EGF riboprobe demonstrating absent hybridisation. Magnification × 200

Fig. 8. Photomicrograph of kidney section treated with anti-EGF antibody pre-incubated with EGF peptide demonstrating no positive staining. Magnification × 100

induction of experimental diabetes [33]. These molecules may have a role in the modulation of EGF expression as may glucose-induced protein kinase C stimulation which has been shown to increase EGF-receptor expression in aortic smooth muscle cells [34].

Tissue growth encompasses increased extracellular matrix to accommodate the increased cell numbers. While EGF has been shown to increase collagen synthesis in kidney cells studied in vitro [35] other growth factors such as transforming growth factor-β (TGF-β) which is also increased in early diabetes-related kidney growth [36] are more likely candidates for the early accumulation of extracellular matrix in diabetes [37]. However, the growth factor milieu of the microenvironment within the kidney may be in part responsible for the differing patterns of growth

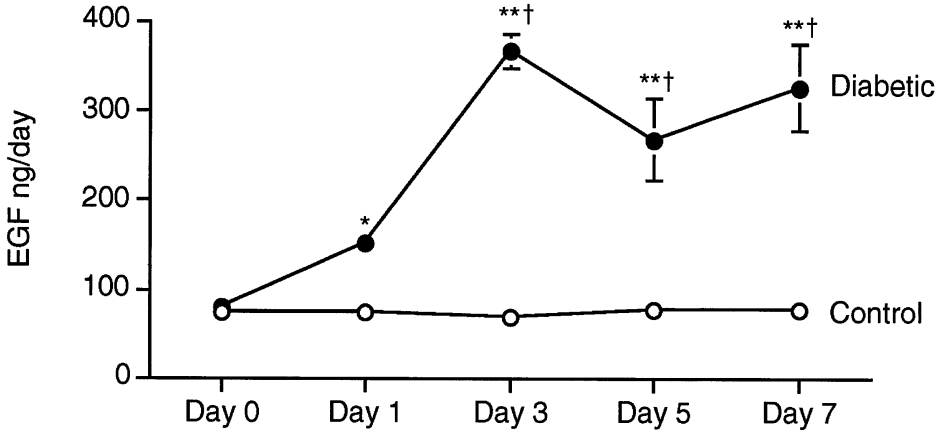

Fig. 9. 9. Urinary excretion of EGF in control (○) and diabetic rats (●). *$p < 0.01$ vs day 0 and control, **$p < 0.001$ vs day 0 and control, † $p < 0.001$ vs day 1

of the tubule compared with the glomerulus. For instance, in the renal tubule which undergoes hyperplasia in diabetes [38] EGF is found abundantly while the generally anti-proliferative cytokine TGF-β is not [39]. The converse applies to the glomerulus which undergoes predominantly hypertrophic changes [38]. Although a study of STZ-diabetic rats did find EGF mRNA in sieved glomeruli using Northern analysis [13] no change in gene expression was noted following the induction of experimental diabetes. These findings are not easily understood in the context of in situ studies in the present report and other studies in which EGF mRNA was not found in the glomerulus of the mouse [40] or in humans [41].

Increased kidney mass found at 36 h following the induction of STZ diabetes continues rapidly for the first week and then slows but continues for up to 6 weeks [3]. Thus the previously reported increase in urinary EGF reported at 2 days of diabetes [12] may have been a consequence of increased kidney size rather than an initiating factor. The present study extends these initial findings by documenting increased EGF mRNA and urinary EGF peptide at 24 h following STZ injection, prior to a change in kidney weight. In humans, renal enlargement is present at diagnosis in insulin-dependent diabetes and does not increase with diabetes duration. Thus the findings of similar levels of urinary EGF in long-term diabetic and control subjects [42] are consistent with EGF being involved in initial kidney growth and later returning to normal levels when growth ceases, as has been described in compensatory renal enlargement following unilateral nephrectomy [11].

The location of EGF and its receptors within the kidney is not completely understood and may change in pathological states. For instance, in control rats EGF is localised immunohistochemically almost exclusively to the distal tubules and collecting duct whereas following nephrotoxic injury substantial EGF immunoreactivity appears in the proximal tubule [43, 44]. In contrast to nephrotoxic injury, experimental diabetes was not associated with any alteration in the sites of EGF expression in the present study. With regard to EGF binding to cells, kinetic analysis has revealed the presence of a low affinity non-saturable kidney tubular lumen uptake mechanism in addition to high affinity binding localised to the basolateral portion of the cell [45]. It is hypothesized that with the increased amount of EGF passing through the renal tubules in early diabetes as found in the present study the luminal uptake mechanism may assume a greater importance in determining EGF action. However, EGF is unlikely to be the sole growth factor involved in kidney growth in early diabetes. Increased gene expression of other growth factors such as platelet-derived growth factor and TGF-β have been found in the glomerulus in experimental diabetes [13]. Proliferation in cells of the proximal tubule may reflect the actions of still other growth factors such as the recently described heparin-binding EGF-like growth factor which is increased in STZ-diabetes [46] and has been postulated to stimulate mitogenesis in the proximal tubule by acting as a paracrine growth factor through binding to EGF receptors at that site [47].

In conclusion, induction of experimental diabetes is associated with increased EGF as a result of elevated gene transcription from normal sites within the kidney. In the context of the known biological actions of EGF the findings of the present study are consistent with a role for this growth factor in early diabetes-related kidney growth.

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