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Chronic treatment with epidermal growth factor stimulates growth of the urinary tract in the rat

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Abstract Twenty-four male Wistar rats, 8 weeks old, were allocated into three groups and treated with human recombinant epidermal growth factor (EGF) administered subcutaneously in doses of 0, 30, and 150 μg/kg per day for 4 weeks. Blood sampling was done every 2nd week and urine sampling was done for 2 consecutive days every week. The most striking finding was that the ureters were dose dependently enlarged, due to growth of all layers of the ureteric wall. The urothelium of the bladder showed considerable hyperplasticity with a widening of the basal proliferative compartment and a normal differentiation pattern as observed by the expression of carbohydrate epitopes, characterized with lectinohistochemistry. Blood examination revealed a decrease in blood haemoglobin concentration and a slight increase in serum creatinine concentration in the high-dose group. There were no effects of EGF on the urinary excretion of electrolytes, proteins, and endogenous EGF.

Key words Differentiation · Lectins · Renal function · Ureter · Urothelium

Introduction

Epidermal growth factor (EGF) is synthesized in large amounts in the kidneys [29], from where it is secreted in an exocrine manner to the urine [10]. Effects of EGF on renal metabolism [7], electrolyte handling [5], and cell renewal have been explored [24], and a trophic effect of urinary EGF on the urothelium has been proposed [17]. Besides the possible physiological implications, the importance of the EGF growth factor system in urothelial neoplasms is well established as recently reviewed by Neal [20].

Recombinant human EGF is now available, and the benefits of systemic EGF treatment for accelerating renal repair after experimental damage have been explored [1, 25]. We investigated treatment effects on experimental damage in the gastrointestinal tract in pigs and discovered in the course of this work that chronic systemic EGF administration caused a remarkable hyperplasia of the urothelium [32].

The aim of the present investigation was to characterize the effects of chronic systemic EGF administration in the rat on growth of the urinary tract. We focused on the hyperplastic urothelium and characterized the differentiation and maturation by means of lectin binding. Furthermore, we studied the effects of chronic EGF administration on renal electrolyte homeostasis.

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Material and methods

Study animals

The study was conducted on 24 male Wistar rats, approximately 8 weeks old. The animals were housed at 21 °C and fed a standard laboratory diet. The study complied with Danish regulations for care and use of laboratory animals.

Study design

All procedures were carried out on a blinded basis. The animals were allocated into three groups receiving human recombinant EGF (Upstate Biotechnology, N.Y., USA) or solvent (isotonic saline) treatment for 28 days. An injection of 0.5 ml was administered subcutaneously twice daily, giving a total dose of 0 μg/kg per day (group 1, controls, $n = 8$), 30 μg (5 nmol)/kg per day (group 2,

low-dose group, $n = 8$), and 150 μg (25 nmol)/kg per day (group 3, high-dose group, $n = 8$). A single animal in the control group died during ether anaesthesia at 2 weeks of treatment.

Once a week each animal was placed in a metabolic cage for two consecutive 24-h periods. The total intake of water and excretion of urine were recorded during the second 24-h period. Urine samples were stored at −20 °C for later analysis. Urine sampled from days 1, 7, 14, 21, and 28 were analysed for concentrations of sodium, potassium, magnesium, creatinine, protein, rat EGF, human EGF (high-dose group only), and urine from days 14 and 28 in addition for calcium and phosphate. Sodium, potassium, and creatinine were analysed on a Kodak Ektachem 500, magnesium on a Perkin-Elmer 2380 atomic absorption spectrophotometer and calcium and phosphate on a Technicon Chem 1. The protein concentration was measured with a BCA Protein Assay Reagent kit (Pierce). Human and rat EGF were measured using species-specific enzyme-linked immunoadsorbent assays (ELISA) [9, 22]. Blood samples were taken from the orbital plexus after 2 weeks (animals under ether anaesthesia) and after 4 weeks at time of death. Anticoagulated blood (ethylenediaminetetra-acetic acid, EDTA) was analysed in a Coulter Counter T890 for total erythrocyte, leucocyte, and platelet counts and haemoglobin, mean cellular haemoglobin concentration, mean erythrocyte cellular volume, and haematocrit. Serum was separated from blood collected without anticoagulant. Serum concentrations of creatinine, sodium, potassium, calcium, phosphate, alkaline phosphatases, and bilirubin were analysed on a Technicon Chem-1. The creatinine clearance was calculated at days 14 and 28 based on 24 h urinary excretion of creatinine and serum creatinine concentration.

Histological specimens

After 28 days of treatment the animals were killed under pentobarbital anaesthesia (50 mg/kg intraperitoneally). The right kidney was removed and weighed. The animals were transcardially perfused at a pressure of 120 mmHg with isotonic saline and subsequently with paraformaldehyde (4%). The left kidney, the ureters, and the bladder were removed. (The total kidney weight was defined as the sum of the right and left kidneys.) The tissues were routinely processed, embedded in paraffin, sectioned at 5 μm, and stained with a combination of periodic acid-Schiff, haematoxylin, and aurantia. The kidneys were sectioned perpendicular to the longitudinal axis through the papilla, the ureters perpendicular to the longitudinal axis approximately 5 mm below the renal pelvis. The bladder was sectioned at a randomly chosen location.

The gross appearance of the kidneys was evaluated on a single section. The cross-sectional area (CSA) of the ureter was calculated as follows. The sections were evaluated using a projection microscope with projection to the table (magnification × 322). The sections appeared circular, oval, or slightly compressed. We assumed that the correct configuration of the ureter was circular. The smallest measurable diameter (the muscular coat defined the outer border) was measured and the CSA was computed as $(\text{diameter}/2)^2 \times \pi$. (This procedure underestimated the CSA on the slightly compressed sections, but probably with equal distribution between the groups.) The relative fractions of the CSA of muscle, submucosa, epithelium, and lumen were estimated by applying a test system with regularly arranged points and counting the points falling into the respective layers [6]. The absolute CSA of each component was then computed by multiplying the relative fraction with the total CSA.

The bladder was evaluated for gross appearance including counting the rows of cells of the urothelium. To describe the differentiation and maturation as expressed by carbohydrate epitopes, lectin characterization of bladder sections from the placebo and the high-dose groups was performed, using a previously published method and as summarized in Table 1 [13]. The staining was scored with respect to cytoplasm and membrane of the basal, intermediate, and luminal layers. In the high-dose group the enlarged intermediate layer was subdivided into outer and inner zones.

All specimens for histological examination by point counting, row counting of urothelium, and scoring of lectin stainings were evaluated independently by two observers, and when meaningful a mean was calculated for each observation.

Statistical analysis

Results are expressed as medians and ranges if not otherwise indicated. The Mann-Whitney non-parametric test was employed to test differences between groups. When no differences between the groups were observed, data are either not given or are presented as the total range for all measurements. The level of significance chosen was 0.05.

Results

Histological examination

EGF treatment induced a dose-dependent growth of the ureters (Fig. 1, Table 2). The median CSAs of the

Table 1 Lectins used in this study (*VVA*, *Vicia villosa*, *PNA* Peanut agglutinin, *UEA-1 Ulex europeus*, *WGA* Wheat germ agglutinin). VVA binds to the simple mucin-type *N*-acetylgalactosamine (GalNAc) core structure, which is O-linked to threonine or serine in the peptide core. PNA binds to GalNAc extended with a galactose (Gal), the Thomsen-Fridenreich antigen or T-structure. The binding of WGA indicates longer branchings with carbohydrate subunits of glucoseamine (GlcNAc), and UEA-1 indicates terminal fucose as indicated in the table. The lectins were horseradish peroxidase (HRP) conjugated lectins purchased from Sigma, St. Louis, Mo., USA. The staining was performed as previously described [13]. In brief, the sections were deparaffinized, rehydrated, endogenous peroxidase activity was blocked, and enzymatic deglycosylation of sialic acid residues was performed by neuraminidase treatment (0.1 U/ml, type V, No. N2876, Sigma) of half of the sections for PNA, WGA, and VVA. Colour development was done with 0.04% 3-amino-9-ethyl-carbazole with 0.01% hydrogen peroxide. The nuclei were stained with Mayer's haematoxylin

Lectin	Concentration employed (mg/ml)	Carbohydrate specificity	References
VVA	0.005	GalNAc-R	Tollefsen 1983 [31]
PNA	0.020	Galβ1–3GalNAc-R	Lotan 1975 [15]
UEA-1	0.001	α-2-L-fucose	Matsumoto 1969 [16]
WGA	0.001	$(\beta$1-4GlcNAc$)_n$	Nagata 1974 [19]

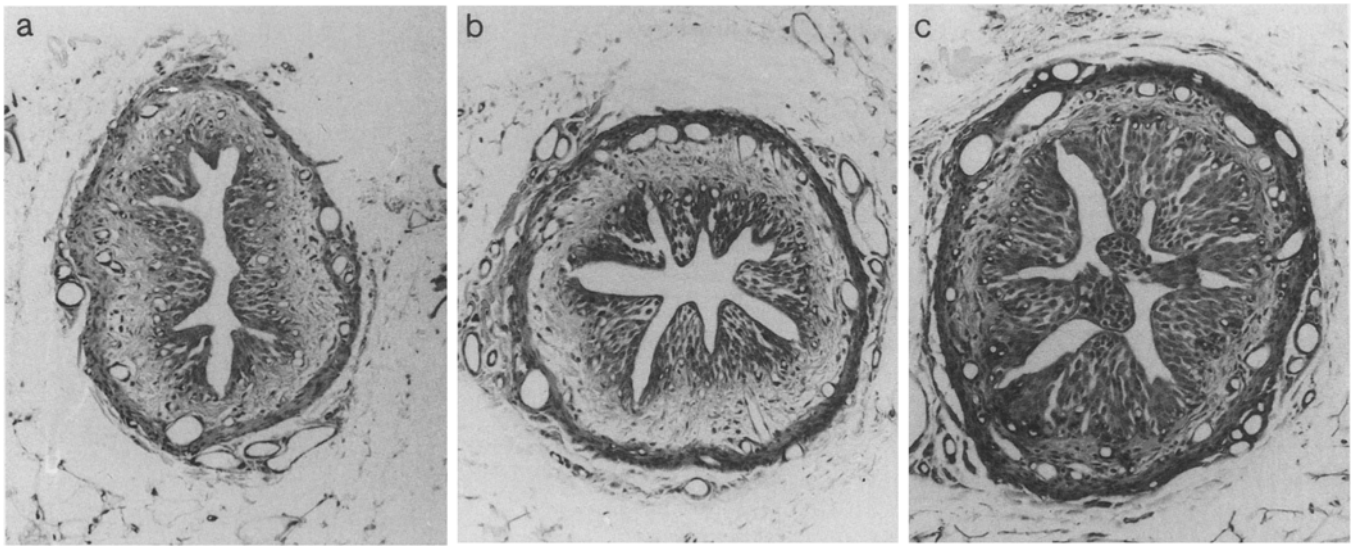

Fig. 1a–c Sections of ureters from rats treated with placebo (**a**), EGF 30 μg/kg per day (**b**), or EGF 150 μg/kg per day (**c**) for 4 weeks. The cross-sectional areas of the different components of the ureteric wall of the control and low- and high-dose groups are given in Table 2, × 123

ureter and its subcomponents were increased in a dose-dependent manner except for the submucosal layer, which was only significantly increased in the high-dose group. The luminal CSA did not change. Quantification with respect to CSA was not performed in the bladder, as this organ was randomly sectioned and was slightly contracted in the high-dose group. However, the transitional epithelium as well as the muscular coat was thickened to a degree, which could not be explained by contraction. There were no differences between groups in weight of the kidneys (total kidney weight ranged from 2.0 to 2.9 g). The histological appearance of the kidneys was normal except for the hyperplastic urothelium at the renal papilla.

The lectins stained the transitional epithelium consistently in the controls and the high-dose group (Fig. 2, Table 3). Peanut agglutinin (PNA) stained the luminal membrane and the immediately underlying cytoplasm. There was no or only scant staining in the intermediate and basal layers. After neuraminidase pretreatment, cytoplasm and membrane staining became apparent in the basal rows of cells in both groups, but were most obvious in the high-dose group. The intensity of cytoplasm and membrane staining declined gradually from the basal layer upward toward the luminal layer, first losing the membrane staining. The staining of the luminal membrane diminished in both groups after neuraminidase treatment. This is rather surprising as neuraminidase treatment usually increases PNA binding. We believe that this appearance is due to competition by the large number of binding sites unmasked in the intermediate and basal layers.

Wheat germ agglutinin (WGA) stained with a granular appearance in the cytoplasm of the basal half of the luminal layer in both groups. Neuraminidase pretreatment did not change these characteristics. In the intermediate cell layer there was, however, sporadic weak membranous staining in the high-dose group. *Vicia villosa* lectin (VVA) marked the whole width of the luminal layer by staining the umbrella cells diffusely. Neuraminidase treatment did not change these characteristics. The luminal cell layer stained accordingly in the high-dose group. After neuraminidase treatment, we observed in this group in addition weak staining of

Table 2 Cross sectional area of ureter in rats treated with placebo, EGF (30 μg/kg per day) (low-dose group), and EGF (150 μg/kg per day) (high-dose group) for 4 weeks. Values are given as medians (ranges) in $\mu m^2 \times 10^3$

	Control group ($n = 7$)	Low-dose group ($n = 8$)	High-dose group ($n = 8$)
Ureter (total CSA)	63 (55–75)	84 (58–130)*	139 (126–186)**,***
Muscular coat	16 (15–18)	21 (16–25)*	31 (22–36)**,***
Submucosa	22 (15–71)	37 (21–50)	58 (35–68)**,***
Urothelium	17 (10–19)	21 (16–40)*	50 (35–78)**,***
Lumen	10 (4–19)	11 (7–17)	11 (8–19)

* $P < 0.05$ compared with control group, ** $P < 0.01$ compared with control group, *** $P < 0.01$ compared with low-dose group

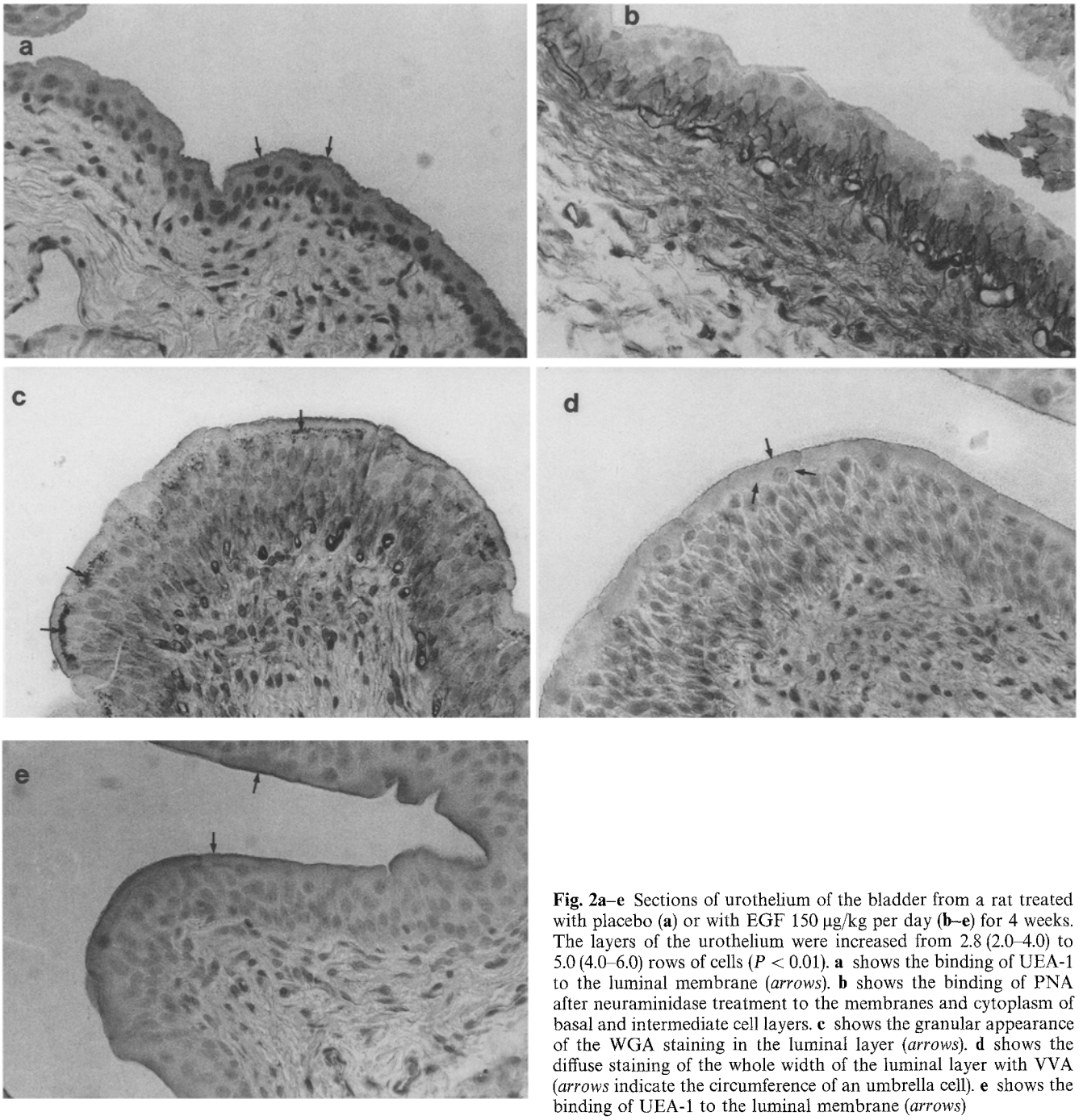

Fig. 2a–e Sections of urothelium of the bladder from a rat treated with placebo (**a**) or with EGF 150 μg/kg per day (**b–e**) for 4 weeks. The layers of the urothelium were increased from 2.8 (2.0–4.0) to 5.0 (4.0–6.0) rows of cells ($P < 0.01$). **a** shows the binding of UEA-1 to the luminal membrane (*arrows*). **b** shows the binding of PNA after neuraminidase treatment to the membranes and cytoplasm of basal and intermediate cell layers. **c** shows the granular appearance of the WGA staining in the luminal layer (*arrows*). **d** shows the diffuse staining of the whole width of the luminal layer with VVA (*arrows* indicate the circumference of an umbrella cell). **e** shows the binding of UEA-1 to the luminal membrane (*arrows*)

the outer cellular row of the intermediate layer in the cytoplasm and on the membranes. *Ulex europeus* lectin (UEA-1) marked the luminal membrane of the luminal cell layer with no differences between the groups. In conclusion, the EGF-induced hyperplastic urothelium expressed the same pattern of lectin binding as normal urothelium, apart from a less sharp demarcation of staining between the three cell layers.

Biochemical examinations of blood, serum, and urine

Haemoglobin concentration in blood was decreased in the high-dose group after 2 weeks of treatment [controls 9.3 (9.0–9.6) and high-dose group 8.5 (8.0–8.8) mmol/l, $P < 0.01$], and stayed at this level until the end of week 4 [controls 9.5 (8.9–9.6)

Table 3 Lectin stainings of bladder urothelium in rats treated with placebo (control group) and in rats treated with EGF 150 μg/kg per day (high-dose group) for 4 weeks. The bladder urothelium is divided into basal, intermediate, and luminal layers. The intermediate layer of the high-dose group was further subdivided into outer and inner zones. The staining was scored with respect to membrane (*M*) and cytoplasm (*C*) (*PNA-N* indicates PNA staining after neuraminidase pretreatment, * indicates neuraminidase pretreatment in the VVA columns)

Lectin	Control group					High-dose group				
	PNA	PNA-N	VVA	WGA	UEA-1	PNA	PNA-N	VVA	WGA	UEA-1
Luminal layer	M				M	M				M
	C	C	C	C		C	C	C	C	
Intermediate layer (outer zone)							M	M*		
							C	C*		
Intermediate layer (inner zone)		M					M			
		C					C			
Basal layer		M					M			
		C					C			

Table 4 Values for haemoglobin, creatinine, urea, and creatinine clearance in rats treated with placebo (controls), EGF (30 μg/kg per day, low-dose group), and EGF (150 μg/kg per day, high-dose group) after 2 and 4 weeks. Values are given as medians (ranges)

Parameter	Group	2 weeks	4 weeks
Haemoglobin (mmol/l)	Placebo	9.3 (9.0–9.6)	9.5 (8.9–9.6)
	Low-dose group	9.2 (8.9–9.9)	9.2 (8.5–9.6)
	High-dose group	8.5 (8.0–8.8)*	8.8 (7.0–9.2)**
Creatinine (μmol/l)	Placebo	61 (54–67)	58 (42–63)
	Low-dose group	60 (43–69)	61 (54–96)
	High-dose group	68 (51–74)	74 (53–84)**
Urea (g/l)	Placebo	7.1 (6.6–9.0)	7.3 (6.0–8.7)
	Low-dose group	6.6 (5.7–8.4)	6.8 (5.3–8.8)
	High-dose group	7.3 (6.0–8.7)	6.8 (5.5–9.6)
Creatinine clearance (l/day)	Placebo	15.5 (13.1–20.0)	23.9 (16.3–28.4)
	Low-dose group	16.0 (13.6–19.3)	19.1 (13.1–25.3)**
	High-dose group	15.8 (11.4–19.6)	20.7 (13.2–19.9)

* $P < 0.05$ compared with control group, ** $P < 0.01$ compared with control group

and high-dose group 8.8 (7.0–9.2) mmol/l, $P < 0.05$], (Table 4). The erythrocyte cell count and haematocrit showed a similar pattern. There were no changes in the other haematological parameters measured.

After 4 weeks of treatment, there was a slight increase in the serum creatinine concentration in the high-dose group [controls 58 (42–62), high-dose group 74 (53–84) μmol/l, $P < 0.05$], and a slight reduction in creatinine clearance in the low-dose group [controls 23 (16.3–28.4), low-dose group 19.1 (13.2–25.3) l/day, $P < 0.05$], (Table 4).

The concentrations of electrolytes and albumin and the other measured concentrations in serum showed minor variations without a characteristic pattern. Overall, these fluctuations (some with a $P < 0.05$ level of significance) seemed sporadic and will not be referred to again in the text.

There were no changes in water intake or of urine excretions during EGF treatment. The water intake was 15–55 ml/24 h, the urine excretion 8–34 ml/24 h, sodium excretion 1020–3648 μmol/24 h, potassium excretion 2840–5742 μmol/24 h, calcium excretion 11–324 μmol/24 h, magnesium excretion 229–549 μmol/24 h, phosphate excretion 1.0–189.2 μmol/24 h, and excretion of protein 54–220 mg/24 h. The measured excretion of endogenous rat EGF was 1418–2808 pmol/24 h and of human recombinant EGF in the high-dose group 1–58 pmol/24 h.

Other systemic effects

All animals thrived throughout the study with no weight differences between the groups. In addition to the changes described in the present paper, we observed a dose-dependent reduction in circulating levels of insulin-like growth factor I (IGF-I) and increased organ weights in the high-dose group in all parts of the gastrointestinal tract [3]. The growth effects observed in the small and large intestines included mucosal growth (epithelium and lamina propria), but in contrast to the growth of the urinary tract no muscular growth [12, 33].

Discussion

The most remarkable findings of this study are the growth-promoting effects of systemic EGF administration on the urothelium and on the other parts of the ureteric and bladder walls in rats.

This study was initiated after we found, by coincidence, that systemic treatment with EGF for 4 weeks induced a four-fold increase in cross-sectional area of the ureter with growth of all wall layers of the ureters and bladder in Goettingen minipigs [32]. The growth in the pigs was more pronounced and differed qualitatively in the presence of glycoconjugate accumulations in the urothelium. The pigs received a dose of 30 μg/kg per day corresponding to the low-dose group of this study. The less pronounced growth effects observed in the rats than in the pigs might be explained by the fact that rats usually need a larger dose per kilogram of a pharmacon for the same effect. Alternatively, there might be species differences in the urothelial response to systemic EGF.

The significance of the EGF growth factor system in bladder neoplasms is well recognized [20]. It is therefore of importance to study whether the EGF-induced hyperplasia has changes associated with malignancy. Carbohydrates are generally considered to be differentiation and maturation markers [21] and lectins have previously been employed for the description of differentiation and maturation of the normal and malignant rat urothelium [13]. The PNA binding is increased especially on cell membranes in association with malignancy in both human [2] and rat urothelium as the Galβ1-3GalNAc epitope is unmasked [14]. However, no dramatic alteration of PNA binding between controls and high-dose EGF animals could be observed. WGA binding (repeated GlcNAc moieties) to rat urothelium shifts from cytoplasm to membranes in association with malignant transformation [13]. However, only a few cells in the intermediate layer of the EGF animals showed membrane staining.

The VVA lectin binds to GalNAc core structures (Tn epitope) with a high affinity. The staining of the luminal cell layer with VVA in both groups of animals indicates the presence of the Tn precursor structure in this cell layer. This is comparable to the presence of immature lacto-series structures Le^a and Le^x in the luminal cell layer of human urothelium [27, 28]. In malignant transformation the immature lactoseries structures in the umbrella cell layer are discovered in all cell layers [26]; however, the VVA staining is still restricted to the luminal cell layer in high-dose EGF animals.

The less sharp demarcation of lectin binding from one cell layer to another in the high-dose EGF animals is interesting, as it could be due to a shortened transit time during EGF challenge, with insufficient time for the cells to modify the carbohydrate chains before the cells move to the next cellular compartment, e.g. the increased expression of PNA binding in the intermediate layer of EGF animals. However, these findings are quite different from the carbohydrate aberrations described during chemical induction of malignancy in the rat bladder [13].

A previous study revealed mitotic activity in the urothelium after intravesical instillation of a small dose of EGF [17]. It was therefore tempting to speculate that an increased urinary load of EGF caused by renal filtration and excretion of the systemically administered EGF initiated the urothelial growth. However, a few nanomoles of endogenous EGF is excreted on a daily basis into the urine; thus the few picomoles of human recombinant EGF excreted in the EGF-treated groups are negligible. The EGF receptors in human urothelium are situated in the basal cell layers [17]. Based on the unchanged excretion of EGF, the assumed receptor location in the rat urothelium, and the growth of the submucosal and muscular layers, we consider the growth effects to be due to an action of EGF from the antiluminal site. It is premature to draw conclusions about the growth sequence of the respective layers, as the induction of growth in one layer of the bladder can cause growth of the neighbouring layers [23].

At present, the physiological and pathophysiologal implications of the findings are uncertain. Growth of the urinary tract in response to urinary overload such as obstruction or hyperdiuresis involves mainly the muscular coat and the connective tissue [18] and to a lesser degree the urothelium. Recent preliminary data have demonstrated upregulated EGF receptor gene transcription proximal to an ureteric obstruction [30]. This finding together with our observations make it of interest to clarify whether EGF receptor stimulation is involved in the growth of the urinary tract in urinary overload.

The most remarkable systemic effect of EGF treatment was the reduced haemoglobin concentration in blood. This finding corroborates our previous findings in pigs, where EGF challenge caused a dose-dependent and reversible anaemia due to an inhibition of bone marrow erythropoiesis [11]. It is difficult to interpret whether the slightly higher concentration of creatinine in serum and the reduced creatinine clearance in the high- and low-dose groups, respectively, reflect decreased renal function, are a statistical fluke, or reflect an altered creatinine metabolism. There are, however, several observations in the literature suggesting that EGF can impair renal function: Harris demonstrated in the rat that intrarenal arterial infusion of 0.5 μg/kg per minute through 30 min decreased the glomerular filtration rate [8], and Goodlad reported that rats treated with approximately 1000 μg/kg per day for 8 days were in poor condition with bloody urine [4]. In the above-mentioned pigs [11], EGF caused reversible increases in the concentrations of creatinine and urea in serum.

In conclusion, chronic systemic EGF treatment for 4 weeks caused considerable ureteric and bladder growth, including the development of a hyperplastic urothelium with a normal differentiative appearance as described by carbohydrate epitope expression. The potent effect on the normal urothelium suggests this epithelium as a target for therapy if it is damaged.

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