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Lars Vinter-Jensen · Karsten Nielsen

The effects of exogenous epidermal growth factor on the developing urinary tract in rats: a stereological description

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Abstract Systemic treatment with epidermal growth factor (EGF) induces growth of all wall layers of the urinary tract in pigs and rats. In this study, we describe the time-dependent growth of the ureter and bladder. Forty-eight female Wistar rats were allocated into five groups receiving EGF treatment (150 μg/kg per day) for 0 (controls), 1, 2, 3 or 4 weeks before being killed. The 24-h urine excretion was increased only in the group treated for 4 weeks with EGF. Measured by a simple infusion device, EGF significantly increased the bladder capacity by more than 50% in all the EGF-treated groups. The volumes of the wall layers of the ureter and bladder were quantified using stereology. After 4 weeks of treatment with EGF, the total volumes of the ureter and bladder were 1.8- and 2.1-fold larger than in the control group (the urothelium was 2.8- and 3.5-fold larger and the muscular coat 1.6- and 1.6-fold larger in the ureter and bladder, respectively). In conclusion, the EGF-induced growth of the urinary tract is characterized by increased bladder capacity, and increased volume of all wall layers – most prominently the urothelium.

Key words Bladder · Growth factors · Stereology · Ureter

Introduction

Epidermal growth factor (EGF) exerts its action via binding to the EGF receptor – an ability shared with other ligands (EGF agonists) of the EGF family [22]. In the urinary tract, EGF is produced in the kidneys and excreted in an exocrine manner to the urine [9]. The EGF receptor and the EGF agonists transforming growth forming alpha (TGFα) and heparin-binding EGF (HB-EGF) are present in all wall layers of the urinary tract of rats [1] and humans [4, 6], and the agonist amphiregulin is present in human urothelium [2]. The physiological significance of the EGF family in the urinary tract is unknown. The importance of the EGF family in growth processes of the urinary tract is suggested by the changed expression of the family members in human urothelial neoplasms [18] and in experimental obstructive growth in the rat [1], and by recent papers by us demonstrating pronounced growth effects of systemic treatment with EGF on all wall layers of the ureter and bladder in Goettingen minipigs and in rats [25, 27].

The EGF-induced growth of the urinary tract represents a new approach to growth of the urinary tract. The growth is different from the growth seen in hyperdiuresis, obstruction, and denervation by having more prominent urothelial proliferation [15]. In the present study we assessed the time-dependent changes induced by 1–4 weeks of treatment with EGF to learn about the sequence of growth of the various wall layers of the urinary tract.

L. Vinter-Jensen (✉)
Department of Clinical Biochemistry,
KH Aarhus University Hospital, Nørrebrogade 44,
DK-8000 Aarhus C, Denmark; Fax: +45 89 49 30 60

L. Vinter-Jensen
Institute for Experimental Clinical Research,
Aarhus University Hospital, Aarhus, Denmark

L. Vinter-Jensen · K. Nielsen
Department of Pathology, Aalborg Hospital, Denmark

Materials and methods

Study animals

The study was conducted in 48 female Wistar rats from our own breed (Department of Pathology, Aalborg Hospital, Denmark), 8 weeks old. The animals were housed individually in cages on white special Spanwall bedding (temperature 21°C, humidity 55 ± 5%, 12-h dark/light cycle). They were fed a standard laboratory diet (Altromin 1314). The study complied with Danish regulations for use of laboratory animals.

Study design

Forty-eight animals were randomly allocated into five groups. They received either subcutaneous injections of solvent (isotonic saline) or human recombinant EGF 150 μg (=25 nmol)/kg per day

(Upstate Biotechnology, New York, N.Y.) twice daily for 4 weeks. Sixteen rats received solvent for 4 weeks, eight rats received solvent for the first 3 weeks and EGF for the last week, eight rats received solvent for the first 2 weeks and EGF for the last 2 weeks, eight rats received solvent for the first week and EGF for the last 3 weeks, and eight rats received EGF during all 4 weeks. All animals were exactly the same age at the beginning and end of the 4-week treatment period. The body weights were determined at the start of the study and after 1, 2, 3 and 4 weeks of treatment. Once a week the animals were placed in a metabolic cage for measurement of water intake and urine excretion for 24 h. After 28 days of treatment the animals were anaesthetized (pentobarbital 50 mg/kg intraperitoneally) and transcardially perfused at a pressure of 120 mmHg with isotonic saline and subsequently with neutral buffered paraformaldehyde (4%).

Bladder capacity

While the animals were perfused with isotonic saline, the bladder capacity was measured by a simple device. A needle connected to a level container (15 cm H_2O) was introduced through the dome of the bladder. The bladder was considered full when the infusion stopped passively (after approximately 2 min) and no bladder contractions were seen on a side-connected pressure measuring device.

Stereological quantification of ureteric and bladder growth

The perfusion-fixed left ureter and the bladder were removed. The volumes of the different wall layers of the ureters were determined using Cavalieri's principle [7]. The length of the ureter was measured and the bladder was weighed. The ureter was divided into proximal, middle and distal thirds. Each third of the ureter was further subdivided into three pieces of the same length; these three pieces were embedded in one block of paraffin. Nine cross-sections (three from each of three blocks) thus represented each ureter. The ureters were cut perpendicular to the longitudinal axis. The bladder was embedded in a single block of paraffin. The bladders were with a random starting point (between 0 and 300 μm from the start of the tissue), cut exhaustively at an intersection distance of 300 μm. The sections were routinely deparaffinized and for stereological examination stained with van Gieson's stain and haematoxylin and eosin (H&E). These sampling procedures ensured that every point in the ureters and bladders had exactly the same chance of being included in the analyses.

The ureters were evaluated using a projection microscope (magnification ×450) and projected on to a grid with regularly arranged points. Points hitting the urothelium, submucosa and muscular coat were counted and the cross-sectional area of each wall component calculated (each point on the grid represented an area of 0.00079 mm^2). The volumes of the wall components for the proximal, middle and distal thirds were thereafter calculated by multiplying the mean cross-sectional area from each third with a third of the length of the ureter. The volume of the total ureter was calculated by summing the volumes from the thirds. The volumes of the wall components of the bladder were estimated as follows. The area of every third section (the first randomly chosen between section 1 and 3) was determined by point counting. The volume fractions of the smooth muscle cells within the muscular coat, the connective tissue within the muscular coat, the submucosa and the urothelium were estimated on each section by counting the number of points falling into the respective wall layers in between five and 75 randomly chosen fields of vision (depending on the size of the section). The volumes of the different wall components of the bladder were thereafter calculated by multiplying the area (areas of sections) weighted volume fractions with the bladder weight assuming a specific density of all tissue components of 1.0 g/cm^3.

Measurements of EGF and protein in urine

Rat EGF was measured with an ELISA in urine treated with trypsin as previously described [10,26]. The amount of human EGF was measured with a previously described ELISA for human EGF [19]. The total protein concentrations were measured with a kit, BCA Protein Assay Reagent (PIERCE).

Statistical analysis

Results are expressed as means and standard error of the mean (SEM). A Mann-Whitney non-paired test was employed for testing differences compared with the control group. Two-tailed tests were employed. The level of significance chosen was 0.05.

Results

General observations

All animals thrived throughout the study. The animals increased in weight from 170 ± 1, 171 ± 1, 175 ± 1, 167 ± 2, and 170 ± 1 g to 199 ± 2, 204 ± 3, 211 ± 2, 204 ± 2, and 219 ± 4 g in the groups treated for 0, 1, 2, 3 and 4 weeks, respectively. The total increase in body weight was significantly larger in the groups treated for 2 and 4 weeks. This is referred to in detail elsewhere [24].

Urine excretion

The 24-h excretion of urine was 10.1 ± 0.3, 11.1 ± 1.5, 11.5 ± 0.8, 9.8 ± 0.4, and 15.1 ± 1.4 ml after 0, 1, 2, 3 and 4 weeks of EGF treatment. After 4 weeks of EGF treatment the urine excretion was significantly greater than in the control group ($P < 0.005$).

EGF in urine

The concentration of rat EGF was unchanged after EGF treatment, but the total excretion of rat EGF was significantly increased after 3 and 4 weeks of EGF treatment. The figures were 1985 ± 65 pmol in the control group, and 1917 ± 204, 2306 ± 203, 2221 ± 119($P < 0.05$) and 2601 ± 190 pmol ($P < 0.01$) in the groups treated for 1, 2, 3 and 4 weeks with EGF. The protein concentration and the total protein excretion were unchanged. The concentration of rat EGF was increased in all the EGF-treated groups from 22 ± 1 pmol in the control group to 28 ± 1 ($P < 0.01$), 28 ± 1 ($P < 0.02$), 30 ± 1 ($P < 0.01$), and 27 ± 1 pmol rat EGF per mg protein ($P < 0.01$) in the groups treated for 1, 2, 3 and 4 weeks with EGF. The concentrations of human EGF in the urine of the EGF treated animals were to low to affect the results.

Bladder capacity

The bladder fillings at 15 cm H_2O were 0.41 ± 0.04, 0.60 ± 0.07, 0.85 ± 0.05, 0.60 ± 0.04 and 0.68 ± 0.05

Table 1 Ureter: volume fractions and volumes (mm^3) of the muscular coat (muscle), the submucosa, the urothelium and the lumen in control rats and in rats treated for 1, 2, 3 or 4 weeks with epidermal growth factor (EGF; 150 μg/kg per day). Data are mean ± SEM

	Controls	EGF treatment for:			
		1 week	2 weeks	3 weeks	4 weeks
Volume fractions					
Muscle	0.27 ± 0.03	0.22 ± 0.01[b]	0.25 ± 0.03[a]	0.24 ± 0.02[a]	0.24 ± 0.01[a]
Submucosa	0.35 ± 0.03	0.41 ± 0.02[b]	0.41 ± 0.03[d]	0.38 ± 0.03[a]	0.38 ± 0.029[a]
Urothelium	0.20 ± 0.02	0.24 ± 0.03[a]	0.26 ± 0.02[d]	0.31 ± 0.03[d]	0.39 ± 0.02[d]
Lumen	0.16 ± 0.07	0.13 ± 0.04	0.11 ± 0.04[a]	0.07 ± 0.03[c]	0.09 ± 0.02[a]
Total volumes					
Total	6.38 ± 0.19	8.15 ± 0.62[a]	8.61 ± 0.39[d]	9.91 ± 0.69d[d]	11.76 ± 0.43[d]
Muscle	1.66 ± 0.05	1.82 ± 0.14	1.99 ± 0.07[c]	2.30 ± 0.15[c]	2.71 ± 0.11[c]
Submucosa	2.22 ± 0.07	3.29 ± 0.29[c]	3.48 ± 0.17[d]	3.76 ± 0.33[d]	4.42 ± 0.17[d]
Urothelium	1.33 ± 0.07	1.98 ± 0.22[a]	2.26 ± 0.13[d]	3.14 ± 0.13[d]	3.57 ± 0.13[d]
Lumen	1.05 ± 0.13	1.06 ± 0.10	0.90 ± 0.11	0.78 ± 0.16	1.08 ± 0.10

Significance levels of EGF groups compared with the control group: [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.005$, [d]$P < 0.001$

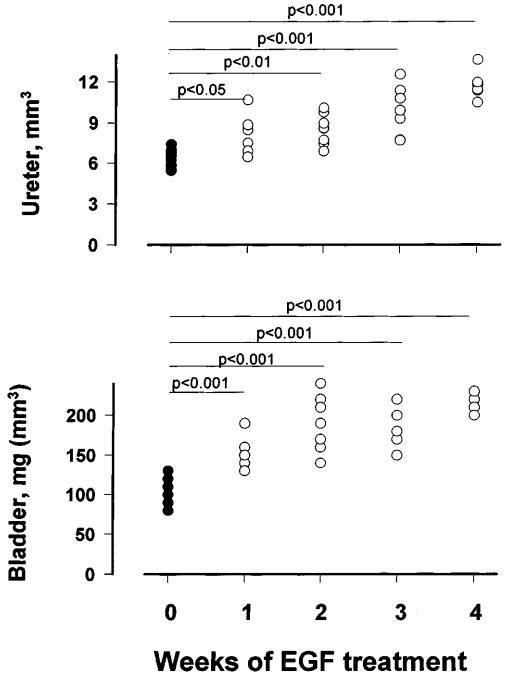

Fig. 1 Scatterplots of the volume of the ureter and weight of the bladder in control rats and in rats treated for 1, 2, 3 and 4 weeks with EGF (150 μg/kg per day). Significance levels for comparison between control group and EGF treated groups are given

ml after 0, 1, 2, 3 and 4 weeks of treatment with EGF. The bladder volumes in all the EGF-treated groups were significantly larger than in the control groups ($P < 0.05$, $P < 0.005$, $P < 0.01$, $P < 0.005$ after 1, 2, 3 and 4 weeks of treatment with EGF).

Stereological quantification of the ureter and bladder growth

The ureter

The length of the ureter was 45.0 ± 0.3, 45.1 ± 0.6, 45.9 ± 0.4, 44.4 ± 0.6, and 46.8 ± 0.5 mm in the groups treated for 0, 1, 2, 3 and 4 weeks with EGF. After 4 weeks of EGF treatment the ureter was significantly longer than in the control group ($P < 0.01$).

The growth in the proximal, middle and distal thirds of the ureter was very similar to the growth of the whole ureter. Only the growth of the whole ureter is therefore described (Fig. 1, Table 1).

For the whole ureter, the volume fractions for the submucosa and urothelium were larger after 1 week of EGF treatment than in the control group whereas the volume fraction of the muscular coat was reduced. After 4 weeks of EGF treatment, the volume fraction of the urothelium was almost doubled. Regarding the total volumes, the ureter after 1 week of EGF treatment was significantly larger than the control ureter and grew thereafter with the duration of the EGF treatment. The urothelium and submucosa grew faster than the muscular coat. These layers were significantly increased after 1 week of EGF treatment whereas the muscular coat was significantly increased after 2 weeks of EGF treatment. After 4 weeks of EGF treatment, the urothelium was increased 2.8-fold, the submucosa 2.0-fold and the muscular coat 1.6-fold compared with the control group. The volume of the lumen remained unchanged.

The bladder

The weight of the bladder was 99 ± 4, 148 ± 8, 190 ± 12, 188 ± 8 and 211 ± 4 mg in the groups treated for 0, 1, 2, 3 and 4 weeks with EGF. The bladder weights in all the EGF-treated groups were significantly larger than in the control group ($P < 0.001$ for all EGF groups).

On the sections there was tissue oedema in the submucosal layer and in the connective tissue between the bundles of smooth muscle cells. We believe that this oedema was caused by our procedure for measuring bladder capacity combined with the perfusion fixation. (In support of this assumption, we did not find similar oedema in our first rat study [27] and in other control

Table 2 Bladder: volume fractions and volumes (= weight in mg) of the smooth muscles within the muscular layer (muscle), connective tissue within muscular coat (connective tissue), the submucosa and the urothelium in control rats and in rats treated for 1, 2 or 4 weeks with EGF (150 μg/kg per day). (Estimations were not performed on rats treated for 3 weeks with EGF). Data are mean ± SEM

	Controls	EGF treatment for:		
		1 week	2 weeks	4 weeks
Volume fractions				
Muscle	0.30 ± 0.01	0.25 ± 0.01[a]	0.28 ± 0.01[b]	0.23 ± 0.02[b]
Connective tissue	0.27 ± 0.01	0.27 ± 0.02	0.26 ± 0.02	0.22 ± 0.02[b]
Submucosa	0.37 ± 0.02	0.41 ± 0.03[a]	0.37 ± 0.02	0.45 ± 0.08[a]
Urothelium	0.06 ± 0.004	0.07 ± 0.004	0.09 ± 0.006[a]	0.11 ± 0.009[a]
Total volumes				
Total	102.1 ± 3.7	145.0 ± 7.79[b]	186.7 ± 15.4[d]	211.3 ± 3.98[d]
Muscle	29.6 ± 1.0	36.3 ± 2.7[a]	53.0 ± 5.5[b]	48.6 ± 4.2[c]
Connective tissue	27.8 ± 1.7	40.0 ± 5.3	48.2 ± 5.0[c]	45.5 ± 3.3[d]
Submucosa	38.1 ± 2.4	59.0 ± 3.8[d]	69.0 ± 6.7[c]	94.6 ± 5.8[d]
Urothelium	6.4 ± 0.4	9.8 ± 0.7[c]	15.7 ± 1.4[c]	22.3 ± 1.9[d]

Significance levels of EGF groups compared with the control group: [a]$P < 0.05$, [b]$P < 0.01$, [c]$P < 0.005$, [d]$P < 0.001$

experiments.) This implies that the volume fractions and volumes of the submucosa and connective tissue within the muscular coat are overestimations. We consider these overestimations of minor importance in the present context.

The bladder was significantly increased in weight after 1 week of treatment with EGF (Fig. 1, Table 2). The weight gain was most prominent in the first 2 weeks of EGF treatment. The volume fractions revealed reduced values for smooth muscle and connective tissue within the muscular coat and increased values for the submucosa and urothelium, in accordance with the most prominent weight increases occurring in the latter two layers. After 1 and 4 weeks of EGF treatment, the urothelium was increased 1.5- and 3.5-fold respectively, the submucosa 1.5- and 2.5-fold, the connective tissue within the muscular coat 1.4- and 1.6-fold and the smooth muscle within the muscular coat 1.2- and 1.6-fold.

Discussion

The major finding of the present paper is that the EGF-induced growth process of the urinary tract first and most prominently involves the urothelium and the submucosa and thereafter and less prominently the muscular coat. The EGF-stimulated growing urothelium most likely had a hypersecretion of mucin-type glycoproteins into the urine, which is referred to in [28].

In our previous study in rats [27], we demonstrated that 4 weeks of treatment with EGF at doses of 30 and 150 μg/kg per day induced growth of all wall layers of the ureter in a dose-dependent manner. In the present study, we have assessed the time-dependent changes. We found that the urothelial layer increased most and the muscular layer least in volume in response to EGF treatment. In these rats we also removed the colon and found (with stereological techniques) that the mucosa was significantly increased after 1 week of EGF treatment, the submucosa after 2 weeks of EGF treatment and the muscular layer after 3 weeks of EGF treatment (P. Kissmeyer-Nielsen and L. Vinter-Jensen, in preparation). The findings in the urinary tract and the colon suggest that the epithelial layer is the most responsive and the muscular layer the least responsive to EGF stimulation in tubular organs. In accordance with this concept, systemically administered human EGF to pigs binds to the basal row of cells of the urothelium [25]. Hypothetically, the effect of EGF on the inner epithelial layers may be the principal event and the growth of the other layers secondary effects [20].

We believe that the direct effect of systemic EGF on the urothelium explains the main part of the growth, but the growth process is probably more complex. All wall layers of the urinary tract in rats and humans have EGF receptors [1, 4] and are therefore targets for systemic EGF. EGF may also induce hyperdiuresis [21]. In the present study, we found significant hyperdiuresis only in the rats treated for 4 weeks with EGF. In a more recent experiment, where we treated female rats with the same dose of EGF for 7 days and collected urine on days 2, 5 and 7, we found significant hyperdiuresis (Vinter-Jensen et al., unpublished data) in accordance with the finding of increased bladder capacity. Furthermore, EGF is synthesized in kidneys and a few per cent of systemically administered EGF is excreted into the urine. It should therefore be determined whether increased EGF in the urine contributed to the growth. We measured EGF in the urine and found the concentration to be slightly increased after EGF treament, but the total excretion of EGF was either unchanged or slightly increased in the different EGF groups – in accordance with our previous reports [26, 27]. Urinary EGF is therefore considered unimportant in the EGF-induced growth process described in the present paper.

We employed unbiased stereological techniques to obtain measurements of the growth of the urinary tract. In the urothelium the hyperplasia is prominent, but in the muscular coat it is more discreet. The degree of hyperplasia can not be quantified with the stereological techniques applied in this study. By the use of another modern

stereological technique (dissector principle), where cells are counted in thick sections (i.e. 30 μm or thicker) the hyperplasia (and the hypertrophia) can be quantified.

The significance of the EGF family in urothelial malignant growth is well recognized [17]. The growth process most prominently involved the urothelium but we found no malignant changes. Experimental urothelial cancers in rats start with urothelial hyperplasia that becomes malignant after more than 9 weeks [5]. Our observation period was thus too short to judge whether EGF stimulation was carcinogenic. We therefore also examined the urothelium for markers of differentiation and maturation – carbohydrate structures [28]. Carbohydrate structures have previously been used to describe normal, malignant and EGF-stimulated hyperplastic rat urothelium [13, 14, 27]. As in our first study [27], we did not find carbohydrate aberrations similar to those previously described as being associated with cancer [13, 14].

The possible relationship between the EGF-stimulated growing urothelium and neoplastic growth is, however, interesting. It is well established that the expression of the EGF family often is changed in malignant urothelial growth, and that the degree of upregulation of the expression of the EGF receptor relates to tumour stage and aggressiveness [23, 29]. Most studies find that non-transformed urothelial cells in culture grow in an EGF-independent manner [2], whereas EGF is a potent mitogen for transformed urothelial cells, as demonstrated in cell culture systems and in the heterotopically transplanted bladder in rats [11, 16]. In *N*-butyl-*N*-(4-hydroxybutylnitrosamine) (BBN)-induced carcinogesis in rats, TGFα becomes upregulated [8], suggesting an important role of this ligand. This assumption is supported in transfection studies on rat urothelial cells demonstrating that transfection of normal rat urothelial cells with TGFα leads to upregulation of the EGF receptor but with no malignant transformation whereas similar transfection experiments of already transformed urothelial cells produce accelerated tumorogenesis [12]. Thus it seems as though EGF and TGFα in vitro will only stimulate mitotic activity and tumorogenesis if the cells have already been transformed. Close to malignant tumours the urothelium often becomes hyperplastic. This hyperplasia supposedly is due to growth factors produced in the tumour and acting through paracrine pathways [3]. This hyperplasia may be a phenomenon parallel to that observed after systemic EGF.

In conclusion, the urothelial layer is the most responsive and the muscular layer the least responsive layer of the urinary tract to stimulation with systemic EGF. The urothelial growth process is associated with increased secretion of urothelial mucin-type glycoproteins into the urine.

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