Urol Res (1998) 26:97–103

ORIGINAL PAPER

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Glycoproteins in the urothelium and in the urine of the epidermal growth factor induced growing urinary tract in rats

Received: 5 February 1997 / Accepted: 29 October 1997

Abstract Systemic treatment with epidermal growth factor (EGF) induces growth of all wall layers of the urinary tract in pigs and rats. We have previously described that the EGF stimulated urothelium in Goettingen minipigs accumulates glycoproteins. The aim of the present study was to examine and partly characterize glycoproteins in the urothelium and in the urine from rats treated with EGF. Seventy-two female Wistar rats were allocated into five groups receiving EGF treatment (150 μg/kg per day) for 0 (controls), 1, 2, 3 and 4 weeks before being killed. Glycoconjugates were characterized by means of lectins on tissue sections, and using Western blotting, in bladder extracts and in urine. The characterization mostly focused on the expression of the mucin-type core structures T and Tn using the lectins *peanut agglutinin* (PNA) and *Vicia villosa* (VVA) and specific monoclonal antibodies. The thickened EGF-stimulated urothelium retained the normal differentiation pattern as judged from the appearance on electron microscopy and from the expression of carbohydrate structures. Within the urothelium and in the urine there was increased expression of mucin-type glycoproteins suggesting increased urothelial production and excretion of mucin-type glycoproteins. In conclusion, the EGF stimulated hyperplastic urothelium most probably excretes increased amounts of mucin-type glycoproteins to the urine but it retains the normal pattern of differentiation as assessed by lectin characterization.

Key words Bladder · Growth factors · Ureter

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Introduction

Systemic treatment with epidermal growth factor (EGF) leads to growth of the urinary tract in Goettingen minipigs and in rats [26, 28]. The EGF-induced growth of the urinary tract is characterized by growth of all wall layers. In the minipigs, the urothelium is remarkably hyperplastic with accumulations of glycoconjugates, and most likely with secretion of glycoconjugates to the urine [28].

Changes in carbohydrate structures in the urothelium is a common feature in urothelial malignant growth [2, 10, 19]. In several studies it has been demonstrated that carbohydrate epitopes may serve as markers of maturation and differentiation. Especially the mucin-type carbohydrate structures, the T and the Tn epitopes, have received attention [2, 10, 12, 21]. In urothelial malignant growth, the expression of the EGF family members is often increased [17, 20, 30]. It therefore becomes of interest to examine the expression of carbohydrate epitopes in an EGF-induced hyperproliferative urothelium.

However, glycoconjugates or carbohydrate structures participate in multiple cellular functions [6, 23], and changed expression of carbohydrate epitopes may therefore have biological implications other than those related to malignant growth. Carbohydrate structures within the urothelium may serve such diverse functions as cell adhesions and signalling between cells, inhibit attachment or serve as attachment sites for bacteria [1, 13, 24], and they are important for the formation of precipitates in the urine [5, 7].

In the back-to-back paper [27] and in our previous paper [26], we described that EGF treatment in rats induced hyperplasia of the urothelium but without accumulations of glycoconjugates. However, in the urine of the EGF-treated animals, the concentration of glycoproteins was considerably increased. In this paper we describe and partly characterize glycoproteins in the urothelium and in the urine of EGF-treated rats.

Materials and methods

Study animals

The study (2 separate protocols) was conducted in 72 8-week-old female Wistar rats from our own breed (Department of Pathology, Aalborg Hospital, Denmark). The rats were those as published in [27] and in [29]. The study complied with Danish regulations for care and use of laboratory animals.

Study design (protocol 1 and protocol 2)

Protocol 1

Forty-eight rats 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) twice daily for 4 weeks. Sixteen rats received solvent for 4 weeks, eight rats received solvent for the first 3 weeks and EGF the last week, eight rats received solvent for the first 2 weeks and EGF the last 2 weeks, eight rats received solvent for the first week and EGF the last 3 weeks, and eight rats received EGF during all 4 weeks. Urine sampled during the last 24 h before death was employed for glycoprotein analysis. 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%).

Protocol 2

Concurrently with protocol 1, 24 rats were randomly allocated into two groups. They received twice daily for 4 weeks either subcutaneous injections of isotonic saline ($n = 16$) or human recombinant EGF 150 μg/kg per day ($n = 8$). Urine was sampled from these animals, in metabolic cages the 24 h prior to death. After 28 days of treatment the animals were anaesthetized with Ketamine (Ketalar, Parke Davis, Barcelona, Spain) 100 mg/kg and xylosin (Rompun, Bayer, Leverkusen, Germany) 15 mg/kg intramuscularly. Through a midline incision the bladder was exposed, removed, opened, rinsed, weighed and snap frozen in liquid nitrogen. Two bladders from each group were removed for electron microscopic examination.

Electron microscopic examination

To describe the ultrastructure of the urothelium, two bladders from each group filled with 0.7 ml saline were immersion-fixed in 5% glutaraldehyde in 100-mM cacodylate buffer, pH 7.4. Segments of the mid-ventral portion of the bladders were removed, postfixed in 2% OsO_4 in cacodylate buffer, and embedded in Araldite. For light microscopy, 1- to 3-μm-thick sections were cut and stained with toluidine blue. For electron microscopy, 600- to 900-Å-thick sections were cut with diamond knifes and stained with uranyl acetate.

Partial characterization of glycoconjugate epitopes on tissue sections (protocol 1), bladder extracts (protocol 2) and urine (protocols 1 and 2)

We characterized glycoconjugate epitopes by means of the lectins and antibodies described in Table 1.

Tissue sections (protocol 1)

Previous lectin characterizations of urothelia from different Wistar rat strains have provided different descriptions [10, 28]. It was therefore important to examine whether the pattern of lectin binding was similar to our first study [28]. Furthermore, we stained sections with monoclonal antibodies towards the Tn and T epitopes to verify the specificity of lectins vicia villosa (VVA) and peanut agglutinin (PNA). The stainings were performed as described in Table 1 [and in 10, 28].

A partial characterization of glycoproteins in bladder extracts (protocol 2) and in the urine (protocols 1 and 2)

Tissue specimens (5–15 mg) from six placebo-and six EGF-treated animals were homogenized in 50 μl/mg tissue of a SDS buffer (TRIS-Glycine SDS Sample Buffer (Cat no. LC2676 Novex, San Diego, Calif.) (In final dilution approximately 0.06 M TRIS-HCl, pH 6.8, 10% glycerol (vol/vol), SDS 2%, 0.01% bromophenol blue (wt/vol).) Urine was diluted in the same buffer.

The SDS solutions with bladder extract or urine were then boiled for 2 min. SDS-polyacrylamide gel electrophoresis was performed as described by Laemmli [9]. We employed pre-casted 8% and 12% and 4%–15% and 10%–27% gradient TRIS-Glycine gels (Novex). The gels were either stained with Coomassie stain or the proteins were transferred to a nitrocellulose membrane. The nitrocellulose membranes were blocked with 2% Tween 20 (Merck-Schuchardt, München, Germany), washed in TRIS/PBS and incubated overnight with different peroxidase-labelled lectins (Table 1). Some of the membranes were treated with neuraminidase (N'ase, 0.1 U/ml, 1 h, 37°C) before incubation with lectins.
For comparison between the groups, equal amounts of tissue homogenates (equal volumes) or equal amounts of urine proteins were added to the gels. The comparison of the intensity of the

Table 1 Lectins and monoclonal anti-Tn and anti-T antibodies used in this study

Lectin	Concentration employed (mg/ml)	Carbohydrate specificity	References
VVA	0.02	GalNAc-R	[25]
Anti-Tn	1:50 (dilution)	GalNAc-R	unpublished
PNA	0.02	Galβ1-3GalNAc-R	[14]
Anti-T	1:50 (dilution)	Galβ1-3GalNAc-R	unpublished
UEA-1	0.005	α-2-L-fucose	[16]
DBA	0.01	αD-Gal-Nac	[4]
WGA	0.005	$(\beta 1\text{-}4GlcNAc)_n$	[18]

VVA, (*Vicia villosa*), PNA (*Peanut agglutinin*), UEA-1 (*Ulex europaeus*), WGA (*Wheat germ agglutinin*), and DBA, (*Dolichus biflorus*). VVA and anti-Tn bind to the simple mucin-type N-acetylgalactosamine (GalNAC) core structure, which is O-linked to threonine or serine in the peptide. PNA and anti-T bind 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 glucosamine $(\beta 1\text{-}4GlcNAc)_n$, and DBA and UEA-1 indicate terminal *N*-acetylgalactosamine (αD-Gal-Nac) or fucose (α-2-L-fucose). [The lectins were horseradish peroxidase (HRP) conjugated lectins (Sigma); the monoclonal antibodies were anti-Tn antigen (DAKO-HB-Tnl), code no. M898, Lot 072, DAKO, Copenhagen, Denmark.] Lectinohistochemistry was performed as previously described [10, 28]. In brief, the sections were deparaffinized, rehydrated, endogenous peroxidase activity was blocked and enzymatic deglycosylation of sialic acid residues was performed by neuraminidase (N'ase) treatment (0.1 U/ml), type V, No. N2876, Sigma) on half of the sections for VVA, PNA, and WGA (3-amino-9-ethyl-carbazole served as chromogen). The nuclei were stained with Mayers haematoxylin. Immunohistochemistry for the antibodies anti-Tn and anti-T: half of the sections were treated with N'ase as described above. The sections were preincubated with normal rabbit serum (1:20 in TRIS/PBS) and overnight with the monoclonal mouse antibodies against anti-Tn and anti-T. The detection system consisted of the secondary biotin rabbit-anti mouse antibody (DAKO code E0354 1:100 in TRIS-PBS). The ABC complex (Code no. K0355, DAKO) was employed according to the manufacturers prescription.

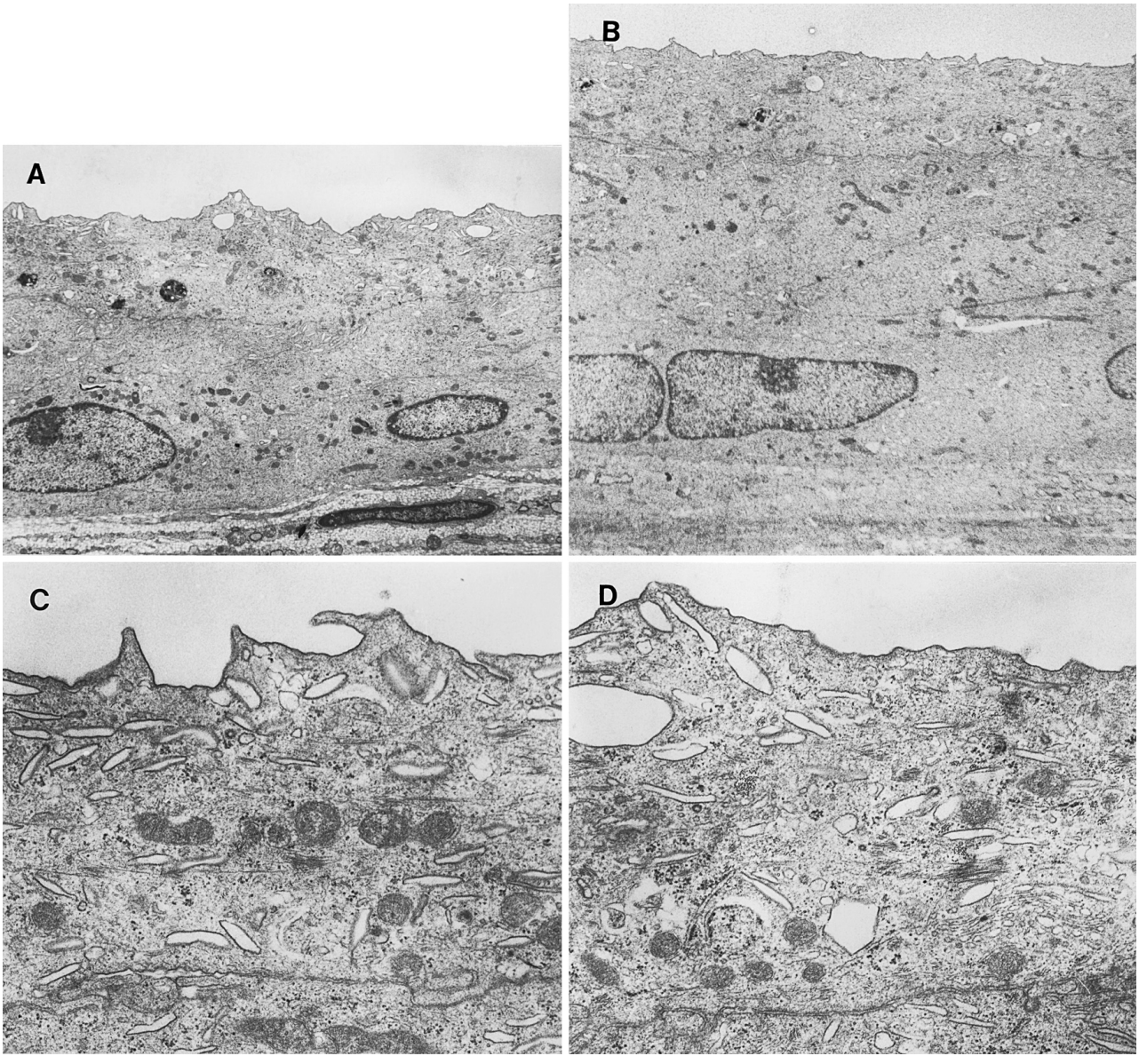

Fig. 1 Electron micrographs of the bladder urothelium from a control rate (**A, C**) and from a rat treated for 4 weeks with epidermal growth factor (EGF), 150 μg/kg per day (**B**, **D**). On magnification ×3600 (**A**, **B**) it is seen that the width of the EGF-stimulated urothelium is increased. On magnification ×18500 (**C**, **D**) it is seen that the width of the umbrella cells are similar in the two groups and that there are no major changes in the organelles

protein bands on the gels relied on a visual judgement. Urine protein was determined with the commercial BCA Protein Assay Reagent kit (PIERCE). The number of animals used for the different purposes are outlined in the Results section.

Results

Bladder weights

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

Electron microscopy

The morphology of the control and EGF stimulated urothelium (4 weeks) is demonstrated in Figure 1. The urothelium from the EGF-stimulated animals appears thicker. On the highest magnification shown, it is seen that the width of the umbrella cells are of approximately the same size, and that the appearances of the asymmetric unit membrane both luminally and in the triangular vesicles in the luminal half of umbrella cells appear similar in the two groups. There are no other major changes in the organelles.

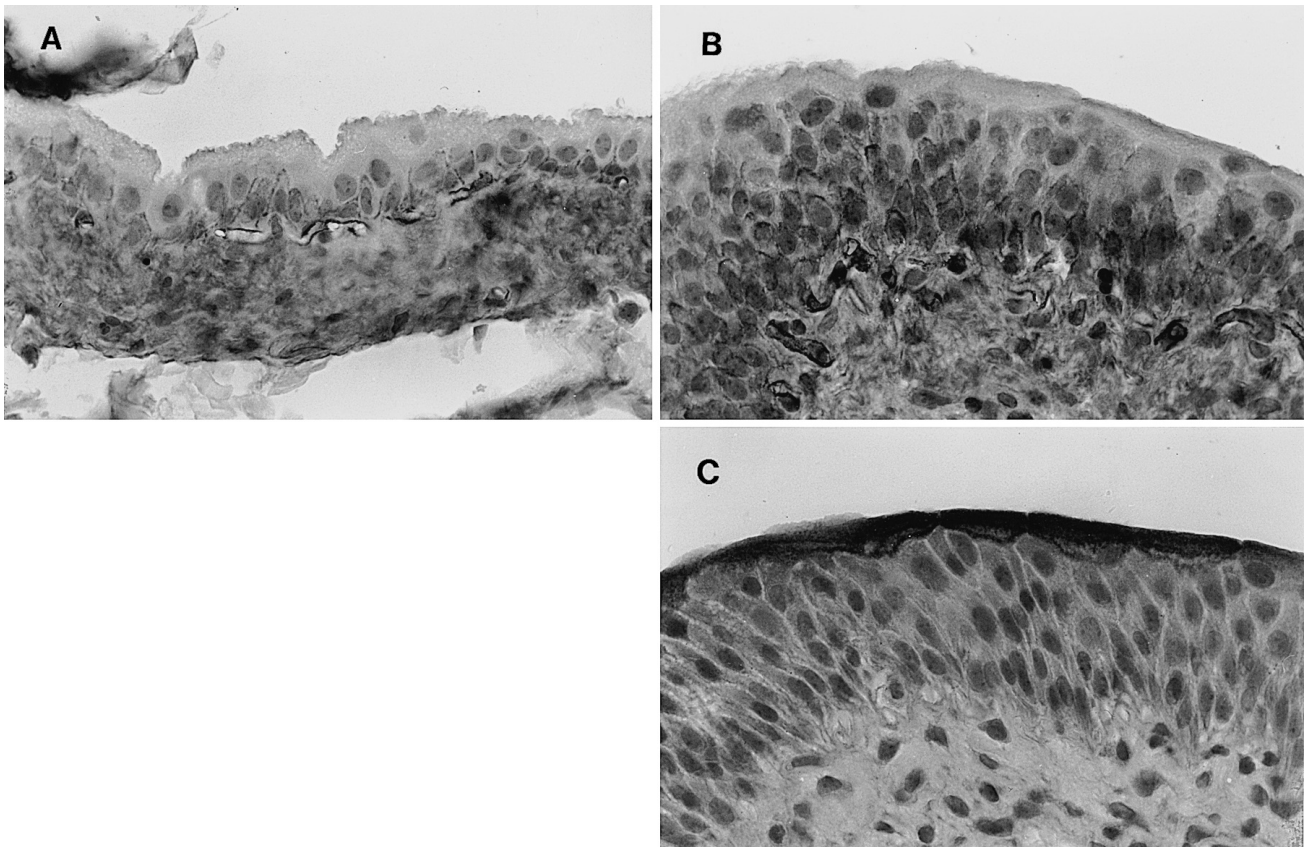

Fig. 2A–C Sections of bladder urothelium from a rat treated with placebo or with EGF 150 μg/kg per day for 4 weeks. The figures demonstrate binding of PNA after neuraminidase pretreatment to a control urothelium (**A**) and to an EGF-stimulated urothelium (**B**). The binding of *Vicia villosa* (VVA) to an EGF-stimulated urothelium is demonstrated on (**C**). The results of the lectinohistochemistry in brief: *peanut agglutinin* PNA bound without neuraminidase (N'ase) treatment to the outer part of the umbrella cells. After N'ase treatment, PNA bound with the greatest intensity to the membranes and cytoplasm of the basal rows of the urothelium in both groups with gradually declining intensity upward into the urothelium. PNA bound to more basal rows in the EGF urothelium than in the control urothelium. Surprisingly, we reconfirmed that the staining of the luminal membrane was lost after N'ase treatment. Below the urothelium, PNA stained after N'ase treatment to the capillaries and connective tissue in the submucosal layer. VVA bound with and without N'ase treatment to the whole width of the umbrella cells, *wheat germ agglutinin* (WGA) stained in both groups with a granular appearance in the cytoplasm of the basal half of the luminal layer and sporadically throughout the urothelium. *Ulex europaeus* (UEA)-1 bound to the luminal membrane and *Dolichus biflorus* (DBA) bound (similarly to VVA) to the whole width of the umbrella cells

Characterization of glycoproteins on bladder and ureter sections

Lectinohistochemistry with PNA, VVA and *Ulex europaeus* (UEA-1) revealed the same results as we have previously described [28]. These findings are briefly outlined in the legend to Fig. 2. Anti-Tn bound (as VVA) to the whole width of the umbrella cell, thereby confirming the specificity of VVA in the urothelium. *Dolichus biflorus* (DBA) bound similarly to the whole width of the umbrella cells. Anti-T did not bind to the urothelium but bound only to the suburothelial capillaries after N'ase pretreatment. The pattern of lectin binding was the same in the urothelium from the bladder and from the ureter.

Characterization of glycoproteins in bladder extracts and in urine

Bladder

We did not find new gross differences on the Coomassie-stained gels. (In a recent paper about smooth muscle proteins, we have described increased intensity of probably the albumin (~66 kDa) and desmin bands (~51 kDa) [29].)

Four control animals and four animals treated with EGF for 4 weeks were run on 8% gels, transferred to membranes and stained with WGA (6 μl bladder extract), VVA, and PNA (both 16 μl bladder extract) after N'ase pretreatment. Wheat germ aggutinin (WGA) bound to so many glycosylated proteins that it was impossible to detect differences. Several bands on the VVA- and PNA-N-stained gels were more intense in the EGF-treated animals. The slightly more intense bands on the VVA-stained gels (Fig. 3A) had molecular weights of about 190, 115, 80, 65, 48 and 30 kDa. The more intense bands on the PNA-stained gels (Fig. 3B) had molecular weights of about 250, 160, 145, 58, 56, 52 and 30 kDa.

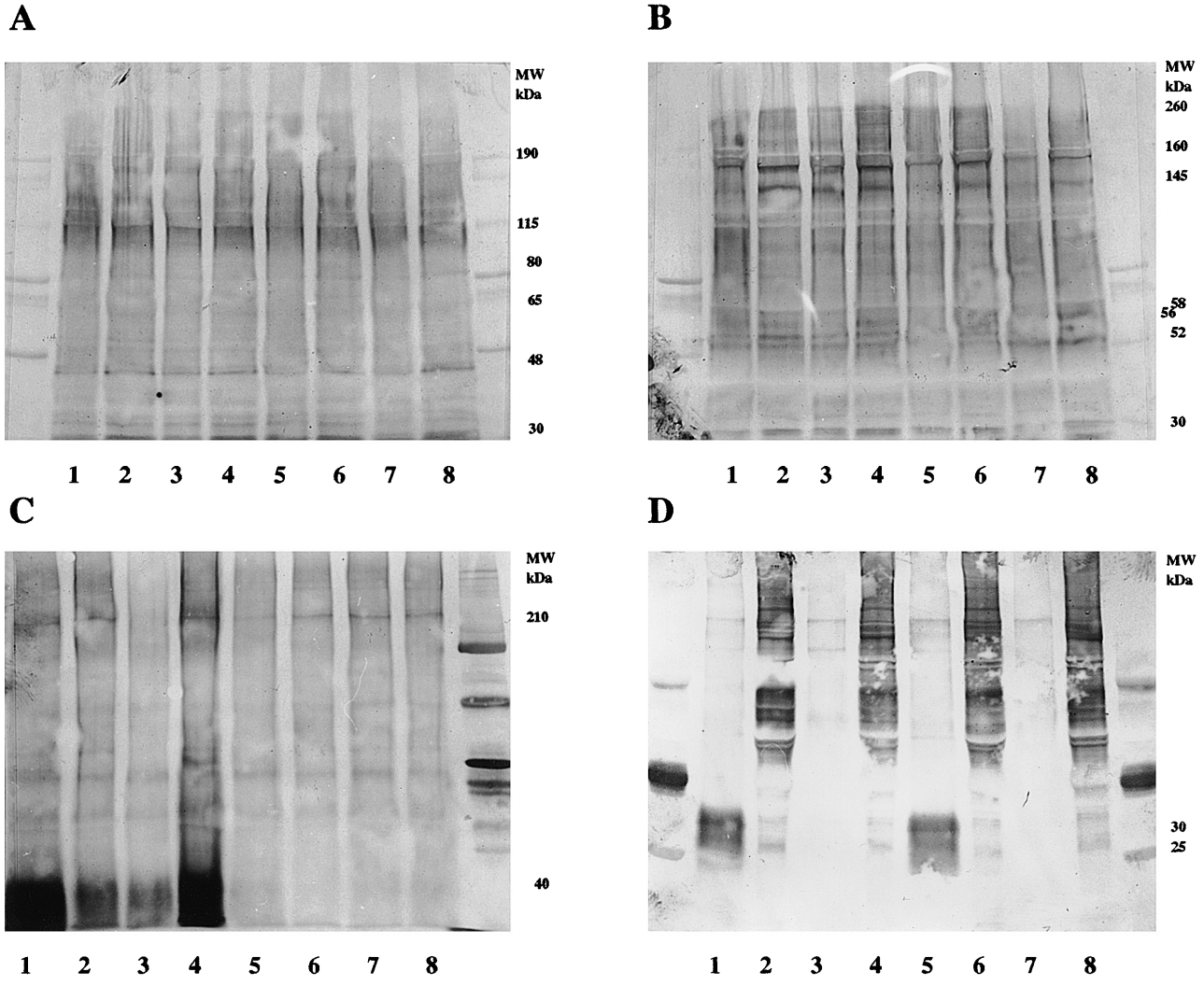

Fig. 3A–D Bladder tissue or urine glycoproteins, from rats treated for 4 weeks with placebo or EGF (150 μg/kg per day), separated by SDS-gel electrophoreses, transferred to nitrocellulose membranes and stained with lectins. Approximate molecular weights of glycoprotein bands which stain more intense in EGF treated animals are indicated. **A** Bladder tissue proteins, 8% SDS gel, 16 μl tissue extract loaded on each lane. The membrane is stained with VVA. Lanes 1, 3, 5, and 7 are from control animals and lanes 2, 4, 6, and 8 from EGF treated animals. **B** Bladder tissue proteins, 8% SDS gel. The membrane is stained with PNA after N'ase pretreatment, 16 μl tissue extract loaded on each lane. Lanes 1, 3, 5, and 7 are from control animals and lanes 2, 4, 6, and 8 from EGF treated animals. **C** Urine proteins, 8% SDS gels, 160 μg urine proteins are loaded on each lane. The membrane is stained with WGA. Lanes 1, 2, 3, and 4 are from EGF animals and 5, 6, 7, and 8 from control animals. **D** Urine and bladder tissue proteins, 12 % gels, 160 μg of urine proteins or 6 μl tissue extract are loaded on the lanes. The membrane is stained with WGA. Lanes 1 and 5 are urine proteins and 2 and 6 bladder tissue from EGF animals. Lanes 3 and 7 are urine proteins and lanes 4 and 8 are bladder tissue from a control animals. On this figure, it is seen that the bands in the smear of urinary glycoproteins below 40 kDa with molecular weights around 30 and 25 kDa also are present in bladder tissue

Urine

WGA was the most sensitive lectin to demonstrate urinary glycoproteins. When 160 μg urinary protein from two animals treated for 0, 1, 2, 3 and 4 weeks were stained with WGA, increased urinary concentrations of glycoproteins were evident after 2 weeks of treatment (not shown). The pattern after 2 weeks EGF was weaker but similar to that after 4 weeks of treatment with EGF, Fig. 3C. Coomassie and WGA staining was performed on urine from approximately 12 control and eight animals treated for 4 weeks with EGF. WGA stained more intensely a smear below 40 kDa, and a smear with a protein band of about 200–220 kDa in the EGF-treated animals. On 12% and 10%–27% gradient gels it was demonstrated that the smear below 40 kDa consisted of two protein bands with molecular weight about 30 and 25 kDa with a smear surrounding. Staining with VVA (with and without N'ase), PNA after N'ase, DBA and UEA-1 was performed on four control and four EGF animals. The Coomassie staining revealed more intense bands of about 230, 80, 38, 30, and 25 kDa in animals treated for 4 weeks with EGF (not shown). The pattern for binding of VVA (with and without N'ase) was weaker but very similar to the pattern of WGA demonstrated in Fig. 3C. The bands around 30 and 25 kDa also bound DBA and UEA-1 (and very weakly PNA-N).

Bladder and urine

When urine proteins and bladder extracts were run on the same gels, the glycosylated proteins of about 30 and 25 kDa in urine were present in bladder extracts (Fig. 3D).

Discussion

The major finding of the present paper is that the EGF-stimulated hyperplastic-urothelium most likely has a hypersecretion of mucin-type glycoproteins to the urine. The significance of the EGF family in urothelial malignant growth is well recognized [17, 20, 30]. The EGF-induced growth of the urinary tract most prominently involved the urothelium but we found no malignant changes as judged by the morphology and expression of carbohydrate epitopes. However, experimental urothelial cancers in rats starts with urothelial hyperplasia which becomes malignant after more than 9 weeks [3]. Our observation period was therefore too short to judge whether EGF stimulation was carcinogenic.

It was therefore important to determine whether markers of differentiation and maturation such as carbohydrate structures indicated a dedifferentiation. Carbohydrate structures have previously been used to describe normal, malignant and EGF-stimulated hyperplastic rat urothelium [10, 11, 28]. We focused mainly on the T and Tn carbohydrate structures since these structures are often changed in urothelial malignant growth [2, 10, 12, 21]. As in our first study [28], we did not find carbohydrate aberrations similar to those previously described to be associated to cancer [2, 10].

The PNA binding after N'ase treatment was widened from one row of cells in the control animals to 3–4 rows of cells after 4 weeks of EGF treatment. We consider this lectin an indicator of immaturity of cells closely time-related to proliferating activity (the proliferative compartment). This concept is in accord with the presence of the T epitope (after N'ase) in the basal proliferative compartment of linear differentiating oral and oesophageal epithelia [8, 15]. The monoclonal antibody to the T antigen bound only to a part of the PNA binding sites in accord with previous descriptions in rats [11]. In accord with the broadened PNA binding zone, we found in the bladder extracts of the EGF animals several more intensely stained PNA binding glycoprotein bands.

The VVA lectin binds to the Tn epitope on mucin type glycoproteins. The specificity of the VVA binding and the specificity of the Tn epitope as a marker of the umbrella cells was confirmed with the monoclonal antibody to the Tn epitope. Our results demonstrate also that mucin type glycoproteins are secreted in increased amounts from the urothelium to the urine during EGF stimulation. The mucin type of these glycoproteins is in accordance with the observation that carbohydrate branchings (apart from anti-T and anti-Tn antibodies) also bound the lectins DBA, WGA and UEA-1 (and PNA weakly). The increased amounts of glycoproteins in the urine was not a matter of exfoliating urothelial cells as the molecular staining of the urine then should have been similar to the molecular staining of the bladder extracts. A renal filtration of these glycoproteins was also unlikely as the protein concentration in the urine was unchanged [27]. It is now of interest in future studies to characterize and identify these glycoproteins, and to establish whether the secretion of these mucin-type glycoproteins has functional implications with respect to formation of urine precipitates and to bacterial adherence, clinical conditions where the composition of glycoproteins are important [1, 5, 7, 13, 22, 24].

In conclusion, the EGF stimulated growing urothelium in the rat has, as assessed with histochemical and biochemical examinations with lectins, a normal pattern of differentiation, increased amounts of PNA and VVA binding mucin type glycoproteins, and increased secretion of glycoproteins to the urine.

Acknowledgements This study was supported by Professor J.C. Christoffersens Mindefond and The Danish Medical Research Council (12-1317). The human recombinant epidermal growth factor was a generous gift from Professor Esam Z. Dajani, International Drug Development Consultants, A division of Mid Gulf USA Inc., Long Grove, Illinois, USA. We thank Ebba Nexø, The Department of Clinical Biochemistry, Aarhus University Hospital and Peter Stubbe Teglbjærg, Department of Pathology, Aalborg Hospital for commenting on the manuscript and for help with the electron microscopic examination, respectively. The technicians Ole Sørensen, Torben Madsen, Kirsten Jensen, Anna Steensgaard and Jette Hansen are thanked for handling the animals, the histological specimens and the biochemical procedures.

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