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Urothelial umbrella cells of human ureter are heterogeneous with respect to their uroplakin composition: different degrees of urothelial maturity in ureter and bladder?

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Dedicated to Werner W. Franke in honor of his 65th birthday

Abstract

Urothelial umbrella cells are characterized by apical, rigid membrane plaques, which contain four major uroplakin proteins (UP Ia, Ib, II and III) forming UPIa/UPII and UPIb/UPIII pairs. These integral membrane proteins are thought to play an important role in maintaining the physical integrity and the permeability barrier function of the urothelium. We asked whether the four uroplakins always coexpress in the entire human lower urinary tract. We stained immunohistochemically (ABC-peroxidase method) paraffin sections of normal human ureter ($n = 18$) and urinary bladder ($n = 10$) using rabbit antibodies against UPIa, UPIb, UPII and UPIII; a recently raised mouse monoclonal antibody (MAb), AU1, and two new MAbs, AU2 and AU3, all against UPIII; and mouse MAbs against umbrella cell-associated cytokeratins CK18 and CK20. Immunoblotting showed that AU1, AU2 and AU3 antibodies all recognized the N-terminal extracellular domain of bovine UPIII. By immunohistochemistry, we found that in $\frac{15}{18}$ cases of human ureter, but in only $\frac{2}{10}$ cases of bladder, groups of normal-looking, CK18-positive umbrella cells lacked both UPIII and UPIb immunostaining. The UPIb/UPIII-negative cells showed either normal or reduced amounts of UPIa and UPII staining. These data were confirmed by double immunofluorescence microscopy. The distribution of the UPIb/UPIII-negative umbrella cells was not correlated with localized urothelial proliferation (Ki-67 staining) or with the distribution pattern of CK20. Similar heterogeneities were observed in bovine but not in mouse ureter. We provide the first evidence that urothelial umbrella cells are heterogeneous as some normal-looking umbrella cells can possess only one, instead of two, uroplakin pairs. This heterogeneity seems more prominent in the urothelium of human ureter than that of bladder. This finding may indicate that ureter urothelium is intrinsically different from bladder urothelium. Alternatively, a single lineage of urothelium may exhibit different phenotypes resulting from extrinsic modulations due to distinct mesenchymal influence and different degrees of pressure and stretch in bladder versus ureter. Additional studies are needed to distinguish these two possibilities and to elucidate the physiological and pathological significance of the observed urothelial and uroplakin heterogeneity.

Keywords: Urothelium; Uroplakins; Cytokeratins; Differentiation; Monoclonal antibody

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Introduction

The superficial umbrella cells of the mammalian urothelium are characterized by numerous rigid-looking urothelial plaques consisting of the asymmetric unit membrane (AUM) (Porter and Bonneville, 1963; Hicks, 1965; Koss, 1969; Warren and Hicks, 1970; Staehelin et al., 1972; Wade and Brisson, 1984; Kachar et al., 1999). The urothelial plaques are composed of a group of integral membrane proteins, the uroplakins (UP). Four major uroplakins have been characterized: the 28-kDa UPIa, 27-kDa UPIb, 15-kDa UPII, and 47-kDa UPIII (Wu et al., 1990, 1994; Yu et al., 1990, 1994; Wu and Sun, 1993; Lin et al., 1994). We have previously shown that the four uroplakins form two pairs consisting of UPIa/UPII and UPIb/UPIII (Wu et al., 1995; Hu et al., 2000; Liang et al., 2001; Tu et al., 2002). A minor component, UPIIIb, which is closely related to UPIII, also pairs with UPIb (Deng et al., 2002). These unique membrane proteins are believed to be involved in mechanical stabilization of the urothelial surface (Staehelin et al., 1972), in the reversible adjustment of the urothelial apical surface area (Porter and Bonneville, 1963; Hicks, 1975; Lewis and de Moura, 1982; Lewis, 1986), and in the permeability barrier function of the urothelium (Hicks, 1975; Lewis, 1986; Chang et al., 1994; Hu et al., 2002). The germline deletion of the UPIII gene in the mouse resulted in major alterations of urothelial morphology, as well as disturbed urothelial function including increased permeability and vesicoureteral reflux (Hu et al., 2000, 2002). Moreover, evidence has been found that UPIa is the urothelial receptor for the uropathogenic *Escherichia coli* (Wu et al., 1996; Zhou et al., 2001) and thus may play a role in the establishment of bacterial infection and in recurrent urinary tract infection (Mulvey et al., 2000).

Immunohistochemical staining showed that uroplakins are associated with the umbrella cells of the urothelial covering of the entire urinary tract including renal pelvis, ureter, bladder and urethra indicating that uroplakins are markers for terminal stages of urothelial differentiation. Until now, we generally assumed that all urothelial umbrella cells possess all four major uroplakins, and that all urothelial plaques are comprised of the same four uroplakins. Using a panel of antibodies to individual uroplakins including three recently established monoclonal antibodies (MAbs) against UPIII, we show here that some of the normal-looking urothelial umbrella cells of human ureter lack the UPIb/UPIII pair. We also demonstrate that this uroplakin heterogeneity is more pronounced in the urothelium of the ureter, than that of the bladder, suggesting that urothelium is biochemically heterogeneous.

Materials and methods

Generation of monoclonal antibodies AU1–AU3

Total (native) bovine asymmetric unit membranes (AUMs) were prepared as described previously (Wu et al., 1990, 1994; Liang et al., 1999). The antigen preparation was suspended in phosphate-buffered saline (PBS) and emulsified with an equal volume of complete Freund′s adjuvant (Sigma), and used for immunization of mice as described previously (Moll et al., 1992). Per injection, 0.25 mg AUM material was applied. Generation and culture of hybridoma cell lines was also performed as described previously (Moll et al., 1992). Hybridoma supernatants were screened by both ELISA, using the AUM preparation as antigen, and by immunohistochemical staining of paraffin sections of formalin-fixed human ureter mucosa (microwave treatment, ABC method; see below).

Production of luminal (N-terminal) and cytoplasmic (C-terminal) fusion peptides of the 47-kDa bovine uroplakin III

The plasmid containing a full-length bovine uroplakin III cDNA (Wu and Sun, 1993) was used as a template in PCR to generate the domain-specific uroplakin III DNA sequences. A sense primer (AGACGAATTCTGTGAACCTCCAGCCCCAAC) and an antisense primer (CGAAGTCGACTCAGTCAGTCAGATCATACCCCCGCTCCGCC) were used to amplify the DNA sequence from the N-terminus of mature UPIII (without signal peptide) to the juxtaposition of the predicted transmembrane domain. The 600-bp PCR product would thus encode a 20-kDa luminal domain of UPIII. In addition, a sense primer (AGACGAATTCCCTCGTGGACAGGGGCGATG) and an antisense primer (GCAAGTCGACTCAGTCAGTCAGTCCTGGAGCTTGCTGG) were used to generate a 190-bp PCR product encoding the entire, 6-kDa cytoplasmic domain. EcoRI and SalI sites were added at the end of the sense and antisense primers, respectively, to facilitate the in-frame cloning of the PCR products into bacterial expression vectors. After PCR amplification, the products were gel-purified and sub-cloned into the pCRII (Invitrogen, Carlsbad, CA). After the two inserts were sequence-verified, they were digested with restriction enzymes and inserted downstream of a GST sequence in the pGEX-4T-3 expression vector (Pharmacia Biotech Inc., Piscataway, NJ). The fusion vectors, pGEX-UPIII-N and pGEX-UPIII-C, were transformed into JM109. A fresh dilute of the bacteria harboring these two vectors were grown at 37 °C to $A_{600} = 0.6$, and the production of the fusion proteins was induced by the addition of isopropyl-beta-D-thiogalactoside (IPTG) to

the growth medium (final concentration 1.0 mM). After 3 h of additional growth at 35 °C, bacteria were pelleted, resuspended, and lysed by sonication. The fusion proteins were purified by passing the bacterial lysate through glutathione–sepharose 4B columns. Control GST protein was also produced by growing and expressing a pGEX-4T-3 starting vector.

Immunoblot analysis and enzymatic deglycosylation

Proteins were electrophoretically separated on a 17% polyacrylamide gel (acrylamide/bisacrylamide ratio 120:1), transferred electrophoretically to nitrocellulose paper, and reacted with various primary antibodies, followed by peroxidase-conjugated goat anti-mouse or goat anti-rabbit IgG antibodies (Woodcock-Mitchell et al., 1982; Eichner et al., 1984). The peroxidase activities were visualized using the enhanced chemiluminescence Western blotting detection system (Pierce, Rockford, IL). For enzymatic deglycosylation, purified bovine AUM was incubated with endoglycosidase H or *N*-glycosidase F (Roche).

Tissues

For the characterization of monoclonal antibodies against uroplakins, paraffin blocks containing various human formalin-fixed tissues were taken from the archival files of the Marburg Institute. Next to ureter and bladder (see below), these tissues included skin, esophagus, stomach, small and large intestine, lung, liver, pancreas, kidney, prostate, mammary gland, and different types of uterine mucosa. In addition, paraffin blocks of transitional cell carcinomas of the urinary bladder ($n = 12$) were used (Riedel et al., 2001).

Cross-sections of 18 human ureters, most of which were resected during nephrectomy because of renal cell carcinoma and therefore comprised the upper portion of the ureter, and 10 specimens of human urinary bladder, resected during biopsy or cystectomy, e.g. because of diverticula, were routinely formalin-fixed and paraffin-embedded. Concerning the ureter samples, the age of the patients ($n = 13$, male; $n = 5$, female) ranged from 7 to 80 years (mean, 58 years). As to the urinary bladder samples, patients ($n = 7$, male; $n = 3$, female) were from 7 to 75 years of age (mean, 52 years). The infantile ureters (7 and 12 years), in one case (7 years) together with the bladder, were removed because of a bladder diverticulum and pyonephrosis, respectively. In addition, one case of a fetal ureter (male; 20 weeks of gestation) was investigated. In each case, a variable number (one to four) of cross-sections of ureter and bladder specimens, respectively, was embedded, mounted on SuperFrost Plus glass slides (Menzel, Braunschweig, Germany) and incubated at 60 °C. Three to four micrometer thick sections were prepared on a microtome. Hematoxylin and eosin (HE) staining was done to exclude any (pre)neoplastic and significant inflammatory changes. All the specimens analyzed showed morphologically regular urothelium. Serial sections were used for immunohistochemistry. Ureter and bladder specimens of cattle and Black SW mice were fixed and processed similarly, as were bladder specimens of sheep and rats.

Immunohistochemistry

ABC method: The avidin–biotin-peroxidase complex (ABC) method was applied using standard protocols (Riedel et al., 2001). Briefly, after deparaffinization and blocking of endogenous peroxidase activity using 1% H_2O_2, sections were treated by microwave oven heating (usually three times for 5 min at 600 W in 10 mM sodium citrate buffer at pH 6.0) for antigen retrieval. Only the sections to be stained for UPIb, CK18 and CK20 were then treated with 0.001% trypsin at 37 °C for 15 min (Riedel et al., 2001). After incubation with normal horse serum (1:10 in PBS), the sections were incubated with the primary antibodies (Table 1) for 1 h at 37 °C in a Sequenza apparatus (Thermo Electron/Shandon, Dreieich, Germany). The MAbs AU1, AU2 and AU3 were applied as hybridoma culture supernatants diluted 1:10 in PBS; dilutions of the rabbit antibodies were 1:1000 to 1:10,000. Negative controls were performed using non-relevant antibodies instead of the specific primary antibody. Bound primary antibodies were detected using biotinylated horse anti-mouse IgG or biotinylated goat anti-rabbit IgG (Vector, Burlingame, CA) at 1:100 dilution in PBS and subsequently the ABC Elite Kit (Vector). The staining reaction was performed using 3,3′-diaminobenzidine (DAB) and H_2O_2. Sections were mildly counterstained with hematoxylin. Photographs were taken using an Olympus AH-3 microscope.

Double immunofluorescence microscopy: Except for omitting the peroxidase blocking step, sections were pretreated as described above, including microwaving. Both primary antibodies [monoclonal AU1 (undiluted hybridoma culture supernatant) combined with rabbit antibodies to UPIa (dilution 1:200), UPIb (dilution 1:2000) or UPII (dilution 1:50), respectively; Table 1] were applied simultaneously at 37 °C for 1 h and subsequently at 4 °C overnight. After rinsing in PBS, the secondary antibodies (FITC-conjugated goat anti-body against mouse immunoglobulins, dilution 1:20, and subsequently Texas Red-conjugated goat antibody against rabbit IgG, dilution 1:60; both from Jackson ImmunoResearch/Dianova, Hamburg, Germany) were applied at room temperature in the dark for 30 min. For control, single immunofluorescence staining using only one primary antibody combined with both secondary

Table 1. List of primary antibodies used

Antibody	Specificity	Source
Anti-UPIa (rabbit)	Uroplakin Ia	Own
Anti-UPIb (rabbit)	Uroplakin Ib	Own
Anti-UPII (rabbit)	Uroplakin II	Own
AU1, AU2, AU3 (monoclonal)	Uroplakin III	Own; available through Progen Biotechnik, Heidelberg, Germany
Anti-UPIII (A) (rabbit)	Uroplakin III	Own
RGE 53 (monoclonal)	Cytokeratin CK18	Euro-Diagnostica, Sanbio/Monosan, Uden, The Netherlands
IT-Ks20.8 (monoclonal)	Cytokeratin CK20	Progen Biotechnik, Heidelberg, Germany
MIB-1 (monoclonal)	Ki-67	Dako, Hamburg, Germany

antibodies was performed to exclude any overlap between the mouse and the rabbit system. Photographs were taken using a Zeiss Axioplan 2 fluorescence microscope.

Evaluation of immunohistochemistry

A positive immunoreaction for the uroplakins was evident as a linear staining along the luminal membrane of the superficial urothelial cells (umbrella cells). The integrity of the luminal cell layer was confirmed by staining of sections serial to those stained for uroplakins using the superficial cell-specific CK18 MAb RGE 53. The staining for the different uroplakin antibodies was compared in serial sections (ABC method) and, in selected cases, by double immunofluorescence microscopy. In four cases of human ureter, the proliferative activity of the urothelial cells was determined by counting Ki-67-positive nuclei in UPIII-positive and UPIII-negative areas of similar size.

Results

Characterization of paraffin-section-reactive MAbs AU1–AU3 against UPIII

In an attempt to produce mouse MAbs that can bind to uroplakins in formalin-fixed human tissues and tumors, we screened for hybridoma clones secreting antibodies able to stain paraffin sections of formalin-fixed material. Mice were immunized with highly purified, native bovine urothelial plaques as a source of uroplakins. Hybridoma clones were initially screened for anti-uroplakin antibodies by ELISA followed by immunohistochemical staining (ABC method) of microwaved paraffin sections of formalin-fixed human ureter mucosa. Three clones (AU1, AU2, AU3), all IgG1, were selected that specifically stained the luminal surface of human urothelial umbrella cells. No detectable staining of any non-urothelial human tissue tested (skin, esophagus, stomach, small and large intestine, lung, liver, pancreas, kidney, prostate, mammary gland, and different types of uterine mucosa) was observed (not shown).

Like established rabbit antibodies against UPIII (Fig. 1a), all three MAbs revealed a very clear-cut and strong apical membrane staining of the urothelial umbrella cells (Fig. 1b–d). Similar staining was obtained with bovine and mouse urothelium (for AU1, see Liang et al., 2001) as well as with rat urothelium (not shown) while sheep urothelium was non-reactive. Urothelium-derived tumors, i.e., the transitional cell carcinomas of human urinary bladder, were immunoreactive in the majority of cases (for AU1, see Riedel et al., 2001), exhibiting uroplakin-typical staining patterns (Moll et al., 1995; Lobban et al., 1998; Yuasa et al., 1998, 1999; Li et al., 1999; Riedel et al., 2001; Xu et al., 2001; Olsburgh et al., 2003). We recently reported that immunoelectron microscopy using MAb AU1 revealed specific immunogold decoration of urothelial plaques and fusiform vesicles of umbrella cells of bovine, rat and mouse urothelium (Liang et al., 2001).

Immunoblotting showed specific reactivity of all three MAbs with bovine UPIII while the other uroplakins present in the urothelial membrane preparations were non-reactive (Fig. 2a and b) (Liang et al., 2001). AU1 also reacted with the 42-kDa degradation product of UPIII after chymotryptic cleavage indicating that the epitope recognized by this MAb resides on the N-terminal extracellular portion of UPIII (Liang et al., 2001). The same was found to be true for MAbs AU2 and AU3 (not shown). Using GST fusion proteins containing either the N-terminal or the C-terminal portion, AU1 recognized in immunoblotting the N-terminal but not the C-terminal fusion protein (Fig. 2c). In immunoblotting on deglycosylated UPIII, the three MAbs were still reactive, indicating their reactivity with the protein backbone but not the sugar residues (Fig. 2d–g). Thus, we have generated three MAbs that specifically react with the N-terminal extracellular portion of UPIII, and that work well for immunohistochemistry done on microwaved paraffin sections of formalin-fixed tissues.

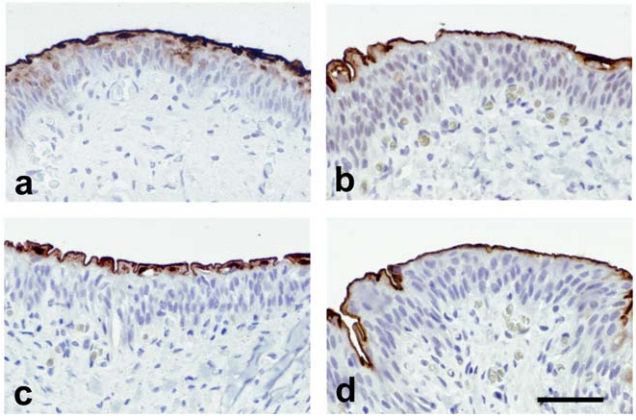


Fig. 1. Immunohistochemical staining of human urothelium using new MAbs against UPIII. Paraffin sections of human bladder were stained with rabbit antibodies (a) and MAbs AU1 (b), AU2 (c) and AU3 (d), all producing a strong linear staining of the luminal membrane of the umbrella cells. Bar, 50 μm.

Differential patterns of uroplakins in human bladder and ureter urothelium

Using the AU1, AU2 and AU3 MAbs and several other antibodies against individual uroplakins (Table 1), we stained immunohistochemically the urothelia of human bladder and ureter. Applying the three UPIII-specific MAbs, we obtained the expected uniform linear superficial membrane staining of the umbrella cells in human bladder urothelium (Fig. 3a). Surprisingly, however, when staining sections of human ureter, we noted with the same antibodies a markedly discontinuous staining of the umbrella cells, with areas exhibiting the typical linear superficial membrane staining sharply alternating with groups or stretches of completely UPIII-negative umbrella cells (Fig. 3b). Morphologically, no difference between the UPIII-positive and the UPIII-negative umbrella cells of the ureter could be recognized. This unexpected finding prompted us to perform a systematic study of multiple cases of human bladder and ureter urothelium on the expression of UPIII and the other uroplakin types. Since a monoclonal antibody to cytokeratin CK18 (clone RGE 53) stained strongly and uniformly the cytoplasm of all superficial umbrella cells, without any staining of the intermediate or basal cells, this antibody provided an excellent, independent marker for the intactness of umbrella cells. Only areas with intact, homogeneous CK18-positive umbrella cell layer were evaluated, in serial sections, for uroplakin staining while areas with artifactually desquamated umbrella cells, easily detected by a lack of CK18 staining, were disregarded. The results of the uroplakin analysis of all human bladder ($n = 10$) and ureter cases ($n = 18$) studied are summarized in Table 2.

Urinary bladder: The urothelium of the urinary bladders often showed marked artificial lesions, but areas exhibiting intact urothelium, as established by staining for CK18, could be identified in all cases. In eight of ten cases a homogeneous expression of all four uroplakins was evident (Table 2). This is illustrated for UPIII in Fig. 3a. Only in two cases, both from the region of the ureter orifice, groups of UPIb-/UPIII-negative superficial cells were present (not shown). As seen in further serial sections of these two cases, reactivity for UPIa and UPII was homogeneously preserved, except for a reduced staining for UPII in the UPIb-/UPIII-reduced area of one of these cases. CK20 was expressed in the cytoplasm of usually the majority of superficial bladder urothelial cells in a heterogeneous pattern; in some cases most superficial cells were CK20-positive (not shown).

Ureter: Of each of the 18 ureter samples, mostly representing the upper (proximal) portion, at least one cross-section was tested using the ABC method. The integrity of the umbrella cell layer was again demonstrated by staining of serial sections for the superficial cell-specific CK18 (Fig. 4a). Three of the ureter specimens showed a homogeneous expression of all four uroplakins in the superficial cell layer (not shown). In contrast, in 15 cases we found a heterogeneous staining pattern for UPIII (Fig. 4b; see also above, Fig. 3b). There was absence of UPIII in a variable fraction of superficial cells, including single cells and small cell groups as well as larger areas, in some cases involving several mucosal plicae and infoldings (see also below, Fig. 6a). There was a rather high degree of variability in extent and distribution of the UPIII-negative umbrella cells. No morphologic difference between UPIII-positive and UPIII-negative umbrella cells, e.g., concerning cell size and height, was observed. There was also no difference in the total thickness of the urothelium or the morphology of the basal cell layer even when long stretches of its overlying umbrella cells were devoid of UPIII. Moreover, no correlation between the distribution pattern of UPIII and the topography of the mucosal plicae (tips vs. infoldings) was noted. All three MAbs, AU1, AU2 and AU3, as well as a rabbit anti-UPIII antibody yielded identical results thus supporting the specificity of the observed umbrella cell heterogeneity. In consecutive serial sections, a very similar heterogeneous staining pattern was observed for UPIb (Fig. 4c), with apparently the same umbrella cells being either positive or negative for both UPIII and UPIb. In serial sections stained for CK18, both the UPIb/UPIII-positive and the UPIb/UPIII-negative cells and cell groups exhibited a similarly strong cytoplasmic decoration (Fig. 4a).

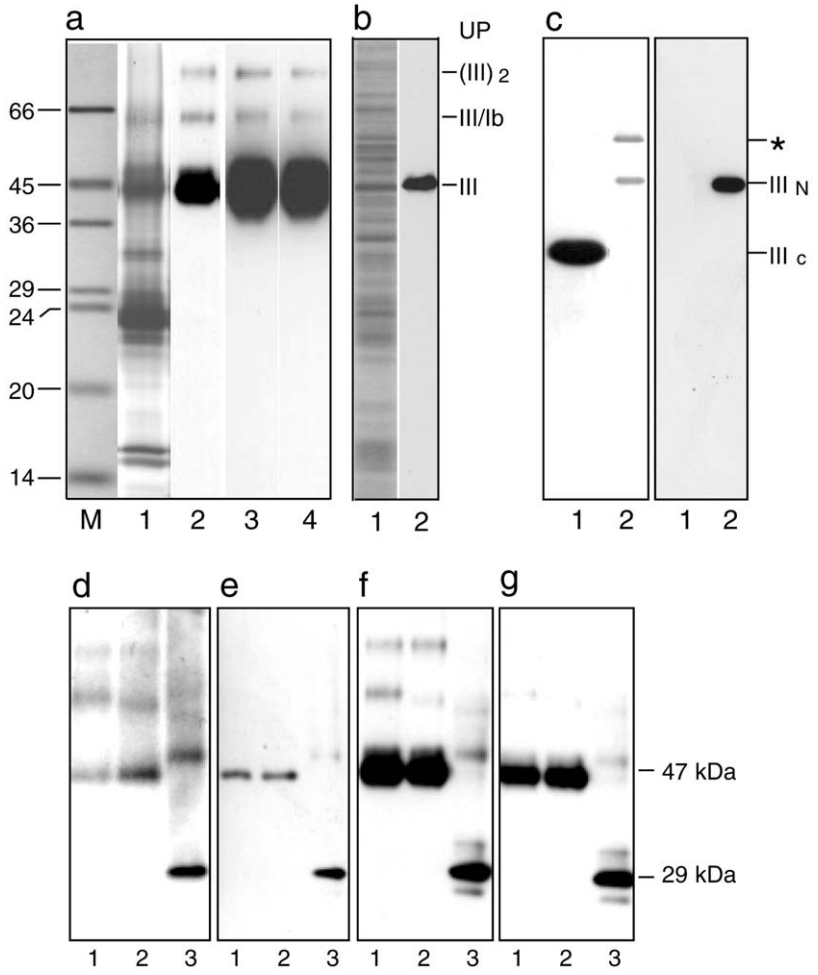


Fig. 2. The specificity of monoclonal antibodies against UPIII. Purified AUMs (a, lanes 1–4), total urothelial membranes from bovine urinary bladders (b, lanes 1 and 2), or GST-fusion proteins of UPIII (c), and deglycosylated uroplakins (d to g, lanes 1–3) were analyzed by SDS-PAGE. Proteins were visualized by silver nitrate staining (a and b, lane 1), by Coomassie blue staining (c, left panel) or immunoblotting using an anti-UPIII monoclonal antibody. M denotes molecular weight markers. (a, b) Note that the monoclonal antibodies specifically react with the 47-kDa UPIII no matter whether purified urothelial plaques (a, lanes 2–4, representing AU1, AU2 and AU3, respectively) or total urothelial membrane extracts (b, lane 2, AU1) were tested. Actually, AU1 specifically reacts with the N terminal (c, lane 2) but not the C terminal (c, lane 1) portion of UPIII in the GST-fusion proteins. There are two cross-reacting bands of higher molecular weight in panel a, lanes 2–4, representing probably the dimers of UPIII (upper band) or UPIII and UPIb (lower band). The asterisk in lane 2 of panel c indicates the dimer of GST. (d–g) Deglycosylation of UPIII. The purified bovine urothelial plaques were treated with buffer (lanes 1), endoglycosidase H (lanes 2) and *N*-glycosidase F (lanes 3). After gel electrophoresis, the proteins were transferred onto nitrocellulose paper. The immunoblots show that AU1 (e), AU2 (f) and AU3 (g) still reacted with UPIII. The same was true for the rabbit antiserum against a UPIII peptide (d). The molecular weight shift of UPIII from 47 to ~29 kDa (lanes 3) indicates that all three MAbs, like the rabbit antiserum, recognize the core protein of UPIII. High-molecular-weight bands again represent dimer formation.

UPIb/UPIII-positive umbrella cells of the ureter urothelium (Fig. 4b and c) consistently showed a co-expression of UPIa and UPII (Fig. 4d and e). Homogeneously UPIb/UPIII-positive cases or stretches were also homogeneously positive for UPIa and UPII. In contrast, with the UPIb/UPIII-negative cells different situations were observed (Table 2). In some cases, UPIa and UPII were present in these cells, showing similar staining intensity as in the UPIb/UPIII-positive areas, and thus were homogeneously expressed (UPIa in $\frac{5}{15}$ cases and UPII in $\frac{3}{15}$ cases; Table 2). In most of the cases, we saw a variable reduction of intensity of the surface membrane staining for UPIa (Fig. 4d) and for UPII (Fig. 4e). Occasionally, single UPIb/UPIII-negative but CK18-positive umbrella cells appeared also to be negative for UPIa/UPII (Fig. 4d, inset). Thus, a small fraction of umbrella cells appears to be completely devoid of uroplakins.

The staining patterns for the different uroplakins as observed by the ABC method on serial sections were

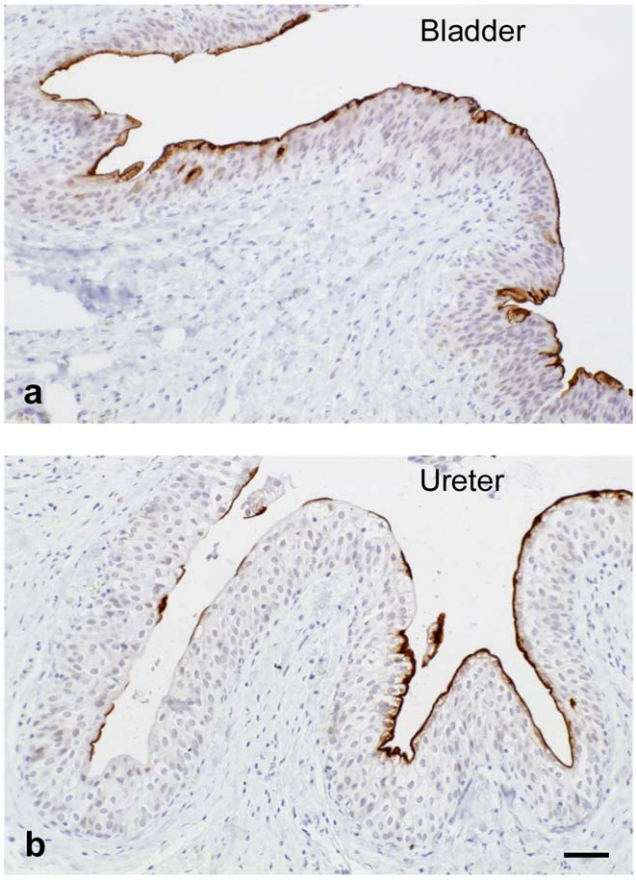


Fig. 3. Expression of UPIII in human bladder and ureter urothelium (paraffin sections, MAb AU1). (a) Bladder urothelium shows a nearly continuous luminal membrane staining of the umbrella cells. (b) In contrast, in ureter urothelium a heterogeneous, clearly discontinuous staining of the umbrella cells for UPIII is observed. Note a continuous UPIII-positive stretch on the right part of the figure but discontinuous staining of single and small groups of umbrella cells on the left part of the figure. The smooth surface in the UPIII-heterogeneous urothelial area suggests integrity of the umbrella cell layer. Bar, 50 μm.

confirmed at the single cell level by double immunofluorescence microscopy of ureter sections. These experiments showed exactly overlapping co-expression patterns for UPIII and UPIb (Fig. 5a and b); importantly, all UPIII-negative umbrella cells were also negative for UPIb. In contrast, when UPIII was compared with UPIa and UPII in such double immunostainings, differential patterns were noted in that most of the UPIII-negative cells (Fig. 5c) were still reactive for UPIa (Fig. 5d) and UPII (not shown). Concerning the UPIII-positive cells (Fig. 5a and c), these cells always co-expressed not only UPIb (Fig. 5b) but also UPIa (Fig. 5d) and UPII (not shown).

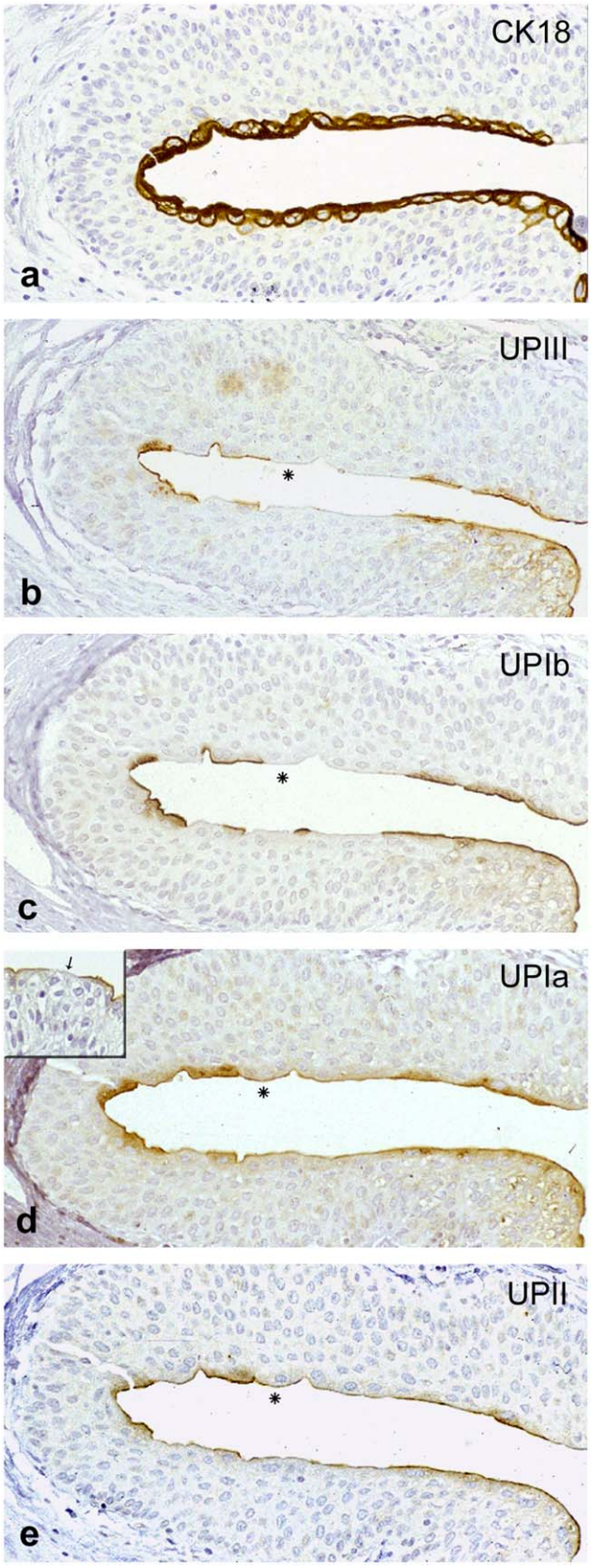
The cytokeratin CK20, another marker of the umbrella cells (Moll et al., 1992), was expressed in the superficial cell layer of the ureter urothelium in a very heterogeneous pattern, typically involving only a few umbrella cells (Fig. 6b), in contrast to the more extended staining in the bladder where usually the majority of and sometimes even most umbrella cells expressed CK20 (not shown). In a few cases of ureter, we saw an expansion of the positive staining to the intermediate cell layer, although, as stated above, there was no evidence of dysplasia. The CK20 pattern was not correlated with the staining pattern for the uroplakins in that among the UPIb/UPIII-positive umbrella cells, some were positive but others were negative for CK20. Conversely, some of the UPIb/UPIII-negative umbrella cells were CK20-positive and others were CK20-negative (Fig. 6a and b). Thus, the staining patterns for CK20 and UPIb/UPIII seemed to be completely independent from each other.

To determine whether the UPIb/UPIII-negative areas of ureter urothelium may undergo hyperplasia, which might be related to a reduced degree of differentiation, we compared the Ki-67 reactivity, in selected cases, in UPIb/UPIII-positive and -negative areas of approximately similar length. We found 0–2 Ki-67-positive

Table 2. Immunohistochemical patterns of uroplakin expression in the superficial urothelial cells of the human ureter and urinary bladder

	n	Uroplakin III		Uroplakin Ib		Uroplakin Ia		Uroplakin II	
		Hom.	Het. neg.	Hom.	Het. neg.	Hom.	Het. red.	Hom.	Het. red.
Ureter									
Homogeneous	3	3		3		3		3	
Heterogeneous (UPIb/UPIII)	15		15		15	5	10	3	12
Urinary bladder									
Homogeneous	8	8		8		8		8	
Heterogeneous (UPIb/UPIII)	2		2		2	2		1	1

n, number of cases (the case of the fetal ureter is not included in this table).
Hom., homogeneous.
Het. red., heterogeneous reduced (subpopulation of superficial cells with reduced staining intensity).
Het. neg., heterogeneous negative (subpopulation of superficial cells with no staining).



nuclei, mostly in the first suprabasal cell layer, sometimes also in the basal cell layer, per infolding (taken as approximate length unit) regardless whether the umbrella cells overlying the region are UPIb/UPIII-positive or negative (not shown). Thus, there was no detectable increase in proliferation in the UPIb/UPIII-negative urothelial stretches.

Concerning the age distribution, the mean age of the patients with homogeneously UPIb/UPIII-positive ureters (the three exceptional cases) was 62 years, which is slightly higher than the mean age of the 15 cases with heterogeneous pattern (57 years). Since there are only three cases in the homogeneous group, additional work is needed to confirm this. Notably, both infantile ureters (7 and 11 years) showed heterogeneous UPIb/UPIII-staining. This indicates that uroplakin heterogeneity is not a phenomenon restricted to an advanced age. On the other hand, in the fetal ureter homogeneous staining for all four uroplakins was observed (results not shown).

Animal urothelium

By immunohistochemistry, the rabbit antibodies against UPIa, UPIb, UPII and UPIII stained the luminal surface of urothelial umbrella cells of bladder in all mammalian species tested. MAb AU1 reacted, in similar fashion, with bovine, rat and mouse urothelium while sheep urothelium was non-reactive; the same was true for MAbs AU2 and AU3.

We were particularly interested in knowing whether heterogeneous immunostaining patterns, as detected in human ureter urothelium with antibody AU1, were also present in animal urothelium. In the cattle, bladder urothelium was homogeneously positive for UPIII but ureter urothelium—like that of human ureter—showed heterogeneous staining for this uroplakin. In contrast, in the mouse and rat, the urothelium of both the bladder

Fig. 4. Differential immunohistochemical staining patterns of uroplakins as detected on serial sections of human ureter (paraffin sections). (a) CK18 (MAb RGE53) shows uniform cytoplasmic staining of the umbrella cells. (b) For UP III (MAb AU1) heterogeneous luminal membrane staining is observed. (c) UPIb exhibits the same heterogeneous pattern as UPIII. Note closely corresponding cells in (b) and (c), being either positive or negative. (d) UPIa is detected in most umbrella cells but the staining intensity is weaker in those cells which are negative for UPIII and UPIb (compare with (b) and (c)). The inset shows another case of human ureter in which one umbrella cell (*arrow*) is negative for UPIa. (e) For UPII again most umbrella cells are stained at the luminal membrane but the UPIb/UPIII-negative cells (see b and c) show fainter staining. Asterisks in (b–e) exemplarily illustrate one umbrella cell which is negative for UPIII (b) and UPIb (c) but faintly positive for UPIa (d) and UPII (e).

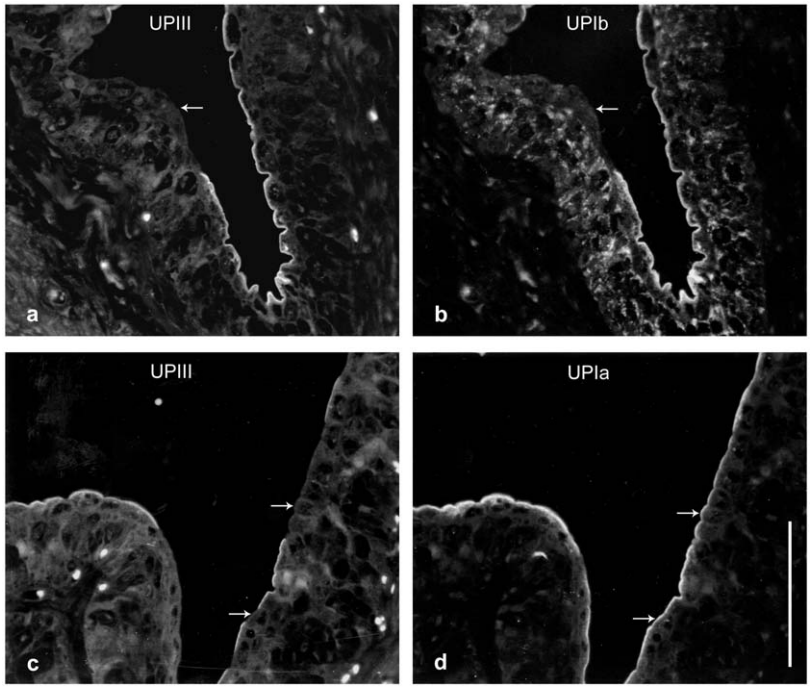


Fig. 5. Double immunofluorescence microscopy of human ureter (paraffin sections) comparing the expression of different uroplakins. MAb AU1 (a, c) was applied simultaneously with rabbit antibodies against UPIb (b) or UPIa (d). (a, b) Completely overlapping staining pattern of UPIII (a) and UPIb (b), with the same umbrella cells showing luminal membrane staining with both antibodies (weaker cytoplasmic staining of lower urothelial cell layers for UPIb was observed in immunofluorescence microscopy but not with the ABC method). Note that other umbrella cells are negative for both antibodies (arrows). (c, d) In contrast, compared with the heterogeneous UPIII staining (c), UPIa decorates all umbrella cells in an apical linear pattern. Some umbrella cells negative for UPIII but positive for UPIa are indicated by arrows. All UPIII-positive umbrella cells (c) coexpressed UPIa (d). The white globules below the urothelium in (a) and (c) represent autofluorescent erythrocytes. Bar, 50 μm.

and the ureter exhibited uniform AU1 staining of umbrella cells (data not shown).

Discussion

Urothelial plaques are major differentiation products of mammalian urothelium, being detectable by electron microscopy in the luminal plasma membrane and in the membrane of cytoplasmic fusiform vesicles of the urothelial umbrella cells (Porter and Bonneville, 1963; Hicks, 1975). We have previously shown that urothelial plaques contain four major integral membrane proteins, occurring as UPIa/UPII and UPIb/UPIII pairs (Wu et al., 1995; Liang et al., 2001; Tu et al., 2002). In all previous immunohistochemical studies performed on urothelium of various species including man and cattle, uroplakins were seen to be homogeneously present over the entire urothelial surface.

The present study presents a detailed and systematic immunohistochemical analysis of all four uroplakins in the same tissue. This study was made possible by the development of a panel of antibodies specific for the individual four major uroplakins. Thereby we confirmed the homogeneity of uroplakin expression in human bladder urothelium in most instances. However, in a majority of cases of human ureter urothelium, a surprising heterogeneity of uroplakin expression among umbrella cells was found. We detected, in most samples of human ureter, a significant subpopulation of urothelial umbrella cells that lack immunostaining for both UPIII and UPIb (Figs. 3b, 4b, c, 5a–c, and 6a). Double immunofluorescence microscopy confirmed the exact co-distribution of the partners UPIII and UPIb at the single cell level (Fig. 5a and b), strongly suggesting the complete absence of this uroplakin pair in this umbrella cell subset. False-negative staining, e.g., due to the masking of antigenic determinants, would appear unlikely since four different antibodies against UPIII (both polyclonal and monoclonal) produced identical results. Interestingly, in most UPIb/UPIII-negative umbrella cells, the other uroplakin pair, UPIa/UPII, seems to be present, albeit often at lower staining intensities, suggesting a reduced UPIa/UPII content. In line with this observation, in a knockout mouse model we found that UPIII ablation leads to loss of the UPIII/UPIb pair which in turn leads to reduced amounts of

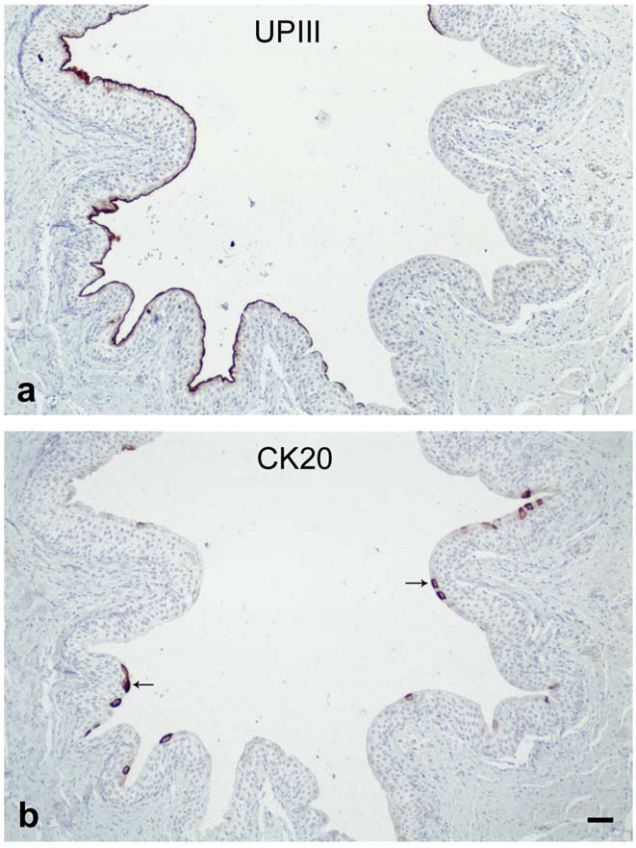


Fig. 6. Comparison of UPIII expression (a; MAb AU1) and CK20 expression (b) on consecutive serial sections of human ureter (paraffin sections). Note completely different staining patterns which apparently are unrelated to each other. For example, the left arrow in (b) denotes a CK20-positive umbrella cell also expressing UPIII (being part of the UPIII-positive stretch in (a)) while the right arrow in (b) points to a CK20-positive umbrella cell negative for UPIII (belonging to the UPIII-negative stretch in (a)). Bar, 50 μm.

UPIa and UPII (Hu et al., 2000). Thus, we have detected the existence of a distinct subpopulation of human urothelial umbrella cells characterized by the presence of only one uroplakin pair.

The parallel behavior of UPIb and UPIII in ureter umbrella cells, being both either present or absent, lends further support to the uroplakin pair concept since there appears to be strict coupling of expression of these two partners in a natural in vivo situation. Our finding that the UPIa/UPII pair can exist in some UPIb/UPIII-negative umbrella cells indicates that the expression of the two uroplakin pairs can be dissociated in vivo. It is noteworthy that even in the absence of UPIb/UPIII, UPIa/UPII can still reach the apical surface as in umbrella cells with a complete uroplakin set. This is consistent with our recent observation that UPIa/UPII and UPIb/UPIII pairs can independently exit from the endoplasmic reticulum to reach the cell surface (Deng et al., 2002; Tu et al., 2002). This is also consistent with our finding that when the mouse UPIII gene was ablated, the remaining UPIa/UPII pair can still be delivered to the apical urothelial surface although in a greatly reduced amount (Hu et al., 2000). Our current observation, that a subpopulation of normal human urothelial umbrella cells make only UPIa/UPII proteins suggests that the expression of UPIb and UPIII is tightly coupled. Our data also demonstrate that the expression of UPIb and UPIII appears to be independently regulated from the expression of UPIa and UPII, suggesting that not all four uroplakin genes are co-regulated. However, this notion has to be confirmed at the mRNA level.

We have proposed previously that the basic building block of the 16-nm AUM particle consists of one copy each of all four major uroplakins (Walz et al., 1995; Min et al., 2002). It is possible that the special type of UPIa/UPII-positive but UPIb/UPIII-negative urothelial umbrella cells described in this study contains only small residual urothelial plaques, like what was observed in the UPIII knockout mouse urothelium (Hu et al., 2000). The formation of these small urothelial plaques in the UPIII knockout mouse urothelium has been interpreted to mean the remaining UPIa/UPII pair may have a lower efficiency to form plaques. Alternatively, the small amount of UPIIIb, which is closely related to UPIII in terms of protein sequence, overall transmembrane topology and its ability to form a heterodimer with UPIb (Deng et al., 2002), may be responsible for the formation of the reduced amounts of unusually small urothelial plaques (Hu et al., 2000; Kong et al., 2004). Actually, recent gene knockout experiments suggest that both major uroplakin pairs are required for the plaque formation (Kong et al., 2004). Future studies by immunoelectron microscopy of ureter urethelium are needed to determine whether some of the superficial umbrella cells (that are UPIb/UPIII-negative) lack the urothelial plaques.

The subpopulation of urothelial umbrella cells with an incomplete set of uroplakins might be considered to be incompletely differentiated. Concerning the reduced uroplakin, one might speculate on possible functional consequences. Uroplakins have been suggested to play a role as permeability barrier (Hu et al., 2000, 2002). If so, umbrella cells containing one uroplakin pair only might be leaky. In fact, increased leakiness has been reported for umbrella cells of mice in which the UPIII gene has been ablated by gene knockout and in which these cells not only lack UPIII but also contain reduced amounts of its partner UPIb (Hu et al., 2000, 2002). However, there are a number of differences between the UPIb/UPIII-negative human umbrella cells and the corresponding cells in the mouse model: in the latter, there is abnormal targeting of UPIb to the basal-lateral cell periphery; there is a profound change of the urothelial surface, including a cuboidal change of the superficial cells; and there is urothelial hyperplasia (Hu et al., 2000; Kong et al., 2004). All these phenotypic changes are

lacking in the stretches of UPIb/UPIII-negative human urothelium as described in the present study. These differences suggest that the lack of two uroplakins in these human umbrella cells is not due to the destruction of the UPIII (or the UPIb) gene as in the knockout mouse but due to another mechanism. This notion is supported by our observations that the number of UPIb/UPIII-negative umbrella cells does not increase with age, thus arguing against an acquired uroplakin gene knockout, and that such cells are already present in infantile ureter. An alternative interpretation is that since there is only a minor population of human ureter umbrella cells lacking UPIb/UPIII, the impact on the cell morphology, proliferation, etc. might be less severe than the uniform UPIII knockout in mouse urothelium.

On the other hand, the absence of any morphological change in the UPIb/UPIII-negative urothelial stretches, which may be quite large and span several mucosal tips and infoldings (see Fig. 6a), suggests that there may be no major functional consequences in ureteral urothelium. It could well be that in this urothelium there are less functional requirements concerning the permeability barrier since, compared with the urinary bladder, the maximal intraluminal pressure in the ureter is lower and the contact time with urine fluid is shorter. In addition, the distension of the ureter is physiologically much less than that of the urinary bladder which undergoes extensive periodic stretch, and thus uroplakins may be less stringently required.

Our finding of the preferential occurrence of UPIb/UPIII-negative umbrella cells in human ureter also indicates that the urothelium of ureter and that of the urinary bladder in humans are phenotypically distinct. Interestingly, another difference between ureter and bladder urothelium concerns the cytoskeletal proteins of the keratins: the cytokeratin CK20, which may be considered to represent another urothelial differentiation marker (Moll et al., 1992), shows a more extended expression among umbrella cells of bladder than among those of the ureter. Although in both locations the CK20 distribution may be heterogeneous, in bladder urothelium often the majority of umbrella cells is CK20-positive while in ureter CK20 may be restricted to a few or single umbrella cells. However, it is notable that on the single cell level there is apparently no relationship between the UPIb/UPIII distribution and the CK20 distribution in the ureter (Fig. 6). The keratin data may be another argument for the view that ureter urothelium is less mature or differentiated than bladder urothelium. The phenotypic differences between ureter and bladder urothelium might also be related to the different embryologic origin (mesodermal for ureter and endodermal for bladder). Interestingly, the only two UPIb/UPIII-heterogeneous samples of bladder urothelium were taken from the ureteral orifice or its immediate surrounding and might therefore be embryologically related to the ureter.

In this study, we have also presented the detailed characterization of a series of novel MAbs against UPIII which, in contrast to the AE31 antibody (Yu et al., 1990), strongly react with paraffin-embedded material, owing to a specially designed screening scheme. Such antibodies are particular valuable for studies in pathology in which uroplakins may be used as highly specific differentiation markers for the identification of the urothelial origin of unknown metastatic carcinomas (Moll et al., 1995; Lobban et al., 1998; Wu et al., 1998; Kaufmann et al., 2000; Gazzaniga et al., 2001; Riedel et al., 2001; Xu et al., 2001; Kageyama et al., 2002; Kreft et al., 2002; Mhawech et al., 2002; Olsburgh et al., 2003; Parker et al., 2003). For such diagnostic purposes, MAb AU1 has already been proven as a very useful reagent to identify urothelial (transitional cell) carcinomas (Kaufmann et al., 2000; Riedel et al., 2001; Parker et al., 2003). Besides normal urothelium, urothelial tumors and Brenner tumors, no UPIII expression has been detected up to now in any other tissue by immunohistochemical methods, underlining the high specificity of this protein for urothelial differentiation. Whether the unexpected expression of the UPIb gene in human corneal epithelium (Adachi et al. 2000), as detected by mRNA analysis, also holds at the protein level remains to be studied. As to transitional cell carcinomas, it will be interesting to clarify whether, analogous to ureter urothelium, focal selective absence of UPIb/UPIII may occur, and, if yes, whether there is any relevance in tumor biology. In fact, we have recently shown that in benign Brenner tumors of the ovary, UPIII is present in somewhat fewer luminal tumor cells than UPIa/UPII (Riedel et al., 2001). Thus, UPIa and UPII might generally represent the more broadly expressed, perhaps "basic" uroplakins, and their expression might be preserved in carcinomas to a higher extent as compared to UPIII. If so, MAbs against UPIa and UPII, which still need to be developed, might be more sensitive than the currently available MAbs AU1–AU3 against UPIII.

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