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Staining patterns of keratins in the human urinary tract

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Summary. The keratins, members of the intermediate filament family, are characteristically expressed in epithelial cells. In the various types of epithelia, the keratin expression pattern is characterized by cell-type specific combinations of the keratin isotypes with a plain pattern in monolayered (simple) epithelia and more complex patterns in stratified and pseudostratified epithelia. Here we demonstrate that the transitional epithelium of the human urinary tract holds an exceptional position between the pseudostratified and stratified epithelia. We show that the simple epithelia keratins 7, 8, 18 and 19 are expressed throughout the whole epithelium as known from pseudostratified epithelia. In addition, we demonstrate expression of keratins 5, 14 and 17, otherwise present in basal cells of multilayered epithelia, and keratins 4 and 13, present in suprabasal areas of non cornified multilayered epithelia. Moreover, we report differences in expression in the various morphological parts of the urinary tract which might be related to their specific functions. Keratin 20, a typical component of the simple epithelia of the digestive tract, is present in bladder and ureter but not in the renal pelvis. Keratin 6, typical for stratified epithelia, is found only in parts of the renal pelvis.

We further show that changes in keratin pattern occur during the development from embryonic to adult bladder urothelium. In contrast to adult tissue, the simple type keratins 7, 8 and 18 are not synthesized in basal embryonic cells. Further, keratin 20, present in cells facing the bladder lumen in adult urothelium, is expressed in all but the basal cells in embryonic bladder.

Key words: Urothelium, Urinary bladder, Keratins, Umbrella cells

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Introduction

The urothelium, lining a major part of the urinary tract, exhibits a complex morphologic structure adapted to its physiological functions and consists in its major part, the urinary bladder, of about five layers of cell nuclei (Petry and Amon, 1966; Jost et al., 1989). The basal cells show an iso- to highly prismatic shape and the intermediate cells exhibit more irregularly formed cell bodies with highly folded lateral cell membranes and long cytoplasmic tails, which are attached to the basal lamina. Increasing expansion of the urothelium, i.e. during filling of the bladder, results in a reduced number of layers of cell nuclei. These morphological changes led to the term transitional epithelium. Of particular importance are the highly specialized apically located so-called umbrella cells, which are well defined from the other cells by a number of morphological criteria (Romih et al., 2005). Umbrella cells are large and multinucleated cells with a diameter of up to 100 µm, allowing them to cover several underlying cells. The luminal cell site of these superficial cells forms the so-called crusta which can be detected by light microscopy (Teutsch, 1977). One report described this crusta to consist of a dense cytoskeletal network made of keratins and actin filaments (Kamada et al., 1997). However, a recent study demonstrated that actin filaments are completely absent from the apical area of differentiated urothelial cells (Romih et al., 1999), indicating that keratins are the exclusive component of the filamentous network responsible for the formation of the crusta.

Physiologic demands on the umbrella cells include the ability to change rapidly cell size and cell diameter according to the filling state of the bladder (Hicks, 1975). The expansion of the apical surface can be achieved particularly by the integration of specific s.c. fusiform or discoid vesicles into the apical plasma membrane, and the reduction of the surface is managed

by internalization of corresponding areas from the plasma membrane (Amano et al., 1988, 1991; Truschel et al., 2002). These physiologic conditions make high requirements on the cytoskeletal keratin network, ensuring strong mechanical support to resist stretching forces without hindering vesicle traffic in the apical cytoplasm. Moreover, variable stress in the different areas of the epithelium and parts of the urinary tract may warrant different keratin patterns and organization. So, rapid changes of volume and shape are of functional importance for the urinary bladder, but not for surface areas of the renal pelvis or the ureter, which represent low pressure tubular organs without reservoir function.

The classification of the urothelium as a stratified (Moll et al., 1988; Jost et al., 1989; Apodaca, 2004; Sun, 2006) or pseudostratified (Bulger, 1988) epithelium is undecided and still controversially discussed. Since the introduction of the human catalogue of cytokeratins (Moll et al., 1982), the identification of these (cyto)keratins can be used to identify and characterize epithelial cell types and tissues, their state of differentiation and also its derived tumors (Moll et al., 1982, 1983; Debus et al., 1982; van Muijen et al., 1986; Moll, 1986). This identification is based on the expression of a characteristic combination of two to ten keratin isotypes in the various epithelial tissues (Franke et al., 1981; Moll, 1993; Chu and Weiss, 2002; Herrmann and Aebi, 2004).

So, for example, monolayered epithelia display a more simple keratin pattern, usually consisting of the isoform pairs keratin 8/18 (K8/18), and keratin 7/19 (K7/19) and in some epithelia, as in human bowel for example, also keratin 20 (K20) (Moll et al., 1990). Stratified epithelia show a more complex pattern with a cell layer-dependent expression of keratins. While keratins 5 and 14 (K5, K14) are characteristically found in the basal cell layers of stratified epithelia, the suprabasal cell layers express the keratin isoforms 1, 10, 2 and 9 (K1, K10, K2, K9) in squamous cornified epithelia, and the isoforms keratin 4, 13 (K4, K13) in non-cornified epithelia. The keratins 6 and 16 (K6, K16) are present mainly in suprabasal cell layers of hyperproliferating tissue, and keratins 3 and 12 (K3, K12) are characteristic for corneal epithelium. While mono- and multilayered epithelia can be characterized by their distinct sets of keratin isoforms (see Table 1), in pseudostratified epithelia characteristically the combination of the keratin isoforms of monolayered and

Table 1. Schematic demonstration of keratin pattern in various epithelia.

	A.														B.				
	Type II						Type I								Type II		Type I		
Keratin (K) No.	1	2e	3	4	5	6	9	10	12	13	14	15	16	17	7	8	18	19	20
Simple Epithelium																			
Liver (hepatocytes)																λ	λ		
Kidney (proximal tubulus)																λ	λ	λ	
Stomach															λ	λ	λ	λ	λ
Colon																λ	λ	λ	λ
Pseudostratified Epithelium																			
Bronchus				λ	λ						λ			λ	λ	λ	λ	λ	
Transitional Epithelium/ Urothelium																			
Bladder				λ	λ					λ	λ			λ	λ	λ	λ	λ	λ
Bladder (embryonal)				λ	λ					λ	λ			λ	λ	λ	λ	λ	λ
Ureter					λ					λ	λ			λ	λ	λ	λ	λ	λ
Pelvis renalis				λ	λ	λ				λ	λ			λ	λ	λ	λ	λ	
Stratified Epithelium																			
Esophagus				λ	λ	λ				λ	λ		λ						
Exocervix				λ	λ	λ				λ	λ	λ	λ						
Epidermis	λ	λ			λ			λ			λ	λ							
Molecular Weight (x10^{-3})	68	65,5	63	59	58	56	64	56	55	54	50	50	48	46	54	52,5	45	40	46

Examples of keratin expression in simple, pseudostratified and stratified epithelia is compared with the keratins found in transitional epithelium/urothelium. Specific keratin expression useful for detailed characterisation of epithelia: red signs; basic keratins: blue signs; additional keratins: grey signs: Large signs: strong expression; small signs: lower expression.

noncornified multilayered epithelia are found (for more details, see reviews (Chu and Weiss, 2002; Moll et al., 2008)).

Our findings add new details on the keratin expression in human urothelium, especially comparing the varying expression pattern in different layers of the bladder with other portions of the urinary tract with distinguishable different physiological roles. Furthermore, we discuss these data, histological criteria obtained by electron and immunofluorescene microscopy to present a characterization and comprehensible classification of the urothelium.

Materials and methods

Tissues

Samples of human bladder and ureter were obtained during routine surgery, immediately snap-frozen in isopentane cooled with liquid nitrogen to about -130°C and stored at -80°C until use. Frozen samples of human renal pelvis and embryonic bladder of the 22nd gestational week were kindly provided by Prof. Gröne (German Cancer Research Center, Heidelberg). Human bladders excised for tumour surgery were immediately used after surgical removal. The expanded bladders were rinsed with ice cold phosphate saline buffer containing EDTA-free proteinase inhibitor (Roche, Mannheim, Germany). Urothelial cells at a distance from the focal pathology leading to bladder excision were carefully scraped off with a scalpel, collected into small volumes of buffer (as above) and either immediately used or frozen and stored at -80°C.

Antibodies

The following primary mouse monoclonal antibodies (mAb) against keratin isotypes were purchased from Progen Biotechnik (Heidelberg, Germany) against: K6, clone Ks6 and KA12; K7, clone RCK 105; K8, clone Ks17.2; K9, a mixture of clones Ks9.70 and Ks9.216; K10, clone RKSE60; K13, clones 2D7 and 1C7; K17, clone Ks17E3; K18, clones Ks18.8.1, Ks18.04, F27.IV and Ks174.1; K20, clones IT Ks20.8 and IT Ks20.10; K2e, clone CK2e; K4, clone 6B10. The polyclonal guinea pig antibody CK5.2 against K5 and the monoclonal antibody against K19 clone Z105.6 were from our own laboratory (Dept of Cell Biology, German Cancer Research Center, Heidelberg, Germany). Monoclonal antibody clones NCL-CK1 against K1, NCL-LL002 against K14 and NCL-CK15 against K15 were obtained from Novocastra Laboratories Ltd. (Newcastle upon Tyne, UK). Secondary antibodies raised in goat against immunoglobulins of mouse and guinea pig and coupled to Alexa 488 and Cy3 were purchased from Dianova (Hamburg, Germany). Primary antibody AU1 to uroplakin III was obtained from Progen Biotechnik (Heidelberg, Germany). An overview of all antibodies used is shown in Table 2.

Table 2. Primary and secondary antibodies used.

specifity	clone	raised in		source
K 6	Ks6, KA12	mouse	monoclonal	Progen Biotechnik
K 7	RCK 105	mouse	monoclonal	Progen Biotechnik
K 8	Ks17.2	mouse	monoclonal	Progen Biotechnik
K 9	Ks9.70/Ks9.216	mouse	monoclonal	Progen Biotechnik
K 10	RKSE60	mouse	monoclonal	Progen Biotechnik
K 13	2D7, 1C7	mouse	monoclonal	Progen Biotechnik
K 17	Ks17E3	mouse	monoclonal	Progen Biotechnik
K 18	Ks18.8.1, Ks18.04,F27IV, Ks174.1	mouse	monoclonal	Progen Biotechnik
K 20	IT Ks20.8, IT Ks20.10	mouse	monoclonal	Progen Biotechnik
K 2e	CK2e	mouse	monoclonal	Progen Biotechnik
K 4	6B10	mouse	monoclonal	Progen Biotechnik
K 5	CK5.2	guinea-pig	polyclonal	Dept. of Cell Biology, DKFZ
K 19	Z105.6	mouse	monoclonal	Dept. of Cell Biology, DKFZ
K 1	NCL-CK1	mouse	monoclonal	Novocastra Lab. Ltd.
K 14	NCL-LL002	mouse	monoclonal	Novocastra Lab. Ltd.
K 15	NCL-CK15	mouse	monoclonal	Novocastra Lab. Ltd.
Uroplakin III	AU1	mouse	monoclonal	Progen Biotechnik

anti	raised in	coupled to	source
mouse	goat	Alexa 488	Dianova
mouse	goat	Cy3	Dianova
guinea-pig	goat	Alexa 488	Dianova
guinea-pig	goat	Cy3	Dianova

Immunofluorescence

3 to 5 µm cryostat sections (Reichert-Jung CM 3000 cryostat, Heidelberg, Germany) were mounted on 3-aminopropyltriethoxysilan-coated slides (SuperFrost*/Plus, Mänzel-Gläser, Braunschweig, Germany), fixed in 100% acetone at -20°C for ten minutes and dried at room temperature overnight. After a brief rinse in PBS, sections were incubated with primary antibodies diluted in PBS for 45 minutes at room temperature, followed by three rinses in PBS (5 min each). Subsequent to incubation in secondary antibody in PBS for 45 minutes, a further three washing steps in PBS were performed and samples were mounted in Fluoromont-G (Southern Biotechnology, Birmingham, USA).

Immunofluorescence was visualized and documented with an Axiophot II (Carl Zeiss, Jena, Germany).

Electron microscopy

For thin section electron microscopy small pieces of human bladder tissue were fixed with 2.5% glutaraldehyde (50 mM sodium cacodylate, pH 7.2, containing 50 mM KCl and 1.25 mM $MgCl_2$ and 1.25 mM $CaCl_2$) at room temperature for 30 minutes. Samples were rinsed repeatedly with 50 mM sodium cacodylate buffer (pH 7.2) and postfixed with ice-cold OsO_4 2% in cacodylate buffer for two hours. After washings with distilled water the samples were stained with 0.5% uranyl acetate in water overnight. Dehydration, embedding and sectioning were performed as described previously (Kartenbeck et al., 1989). Micrographs were taken with an electron microscope EM 910 (Carl Zeiss, Oberkochen, Germany).

Results

Detection of keratins in embryonic and adult bladder, ureter, renal pelvis

The results are presented according to the appearance of keratins in (1) simple/monolayered epithelia, in (2) basal and suprabasal cells of multilayered/squamous non cornified epithelia and in (3) cornified and proliferative epithelia (see also Fig. 1). In the urothelium we distinguish between basal cells with broad contact sites at the basal lamina, superficial cells which face the organs lumen and intermediate cells with cell bodies between these two cell layers and small basal cellular extensions which are in contact with the basal lamina.

(1) The antibodies against keratins K7, 8, 18 and 19 reacted positive throughout all cells of adult bladder, ureter and renal pelvis. In embryonic bladder these antibodies, with the exception of antibodies against K19, did not react with the basal cell layer. The immunostaining for the keratin pair K8/18 (data not shown for K18), and to some extent also for K7 was most intense in cells facing the organs lumen (Fig. 2a-d, 3a,b). Reactions with K20 antibodies were very diverse on the various tissues tested. While in embryonic bladder the vast majority of intermediate and all superficial cells reacted positive (Fig. 3e), immunostaining in adult bladder was restricted only to most, but not all, cells facing the bladder lumen (Fig. 2e). Occasionally these cells showed basally directed cellular extensions, which however, never reached the basal lamina. In the ureter only a few superficial cells with shorter and rarer cytoplasmic tails than in adult bladder showed K20 reaction products (Fig. 4e). The pelvic urothelium tested completely negative for K20 (Fig. 4d). This latter negative result could be verified even by using different antibodies against K20 and by immunoblot reactions using collected epithelial material of the renal pelvis.

(2) The pattern of K5 positive cells within the epithelial layer was mostly similar in embryonic (Fig. 3c) and adult bladder (Fig. 2f) and in the ureter (Fig. 4f). Here we found prominent staining of all superficial cells with positive reactions also in most basal cells, but only very subtle immunoreaction, including also some negative cells, especially in the ureter, in the intermediate located cell bodies. The urothelium of the areas of the investigated renal pelvis revealed a different K5 distribution with staining of all basal cells, but a variable amount of positive intermediate cells and only single positive superficial cells. Detection of the potential partner protein K14 was in all tissues tested mostly poor, restricted to the basal cell layer and lacked the typical filamentous appearance. Using enriched urothelial material of the adult bladder for immunoblot reactions the presence/synthesis of K14, however, could be further confirmed. Keratin 4 antibodies reacted differently in the various tissues. No clear immunostaining was seen in the ureter, some positive basal and intermediate cells were detected in the renal pelvis (Fig. 4b) and adult bladder, while in the embryonic bladder positive cells were seen scattered throughout the whole epithelial layer, occasionally even including some luminal cells. The expression of K13, the potential K4 counterpart, was almost identical in the four tested tissues, with reaction products in all basal and most intermediate cells. Some differences were only seen in the embryonic bladder, with a small amount, and in the renal pelvis with a larger amount of non reactive uppermost located intermediate cells (Fig. 3d).

(3) Keratin 6 was not found in embryonic and adult bladder and in ureter. In renal pelvis, however, all basal and suprabasal cells were positive for K6 (Fig. 4c). Although this positive reaction of the lower urothelial half was generally representative for the K6 distribution, certain areas showed additionally scattered positive intermediate cells. With antibodies against K17, staining of basal cells was seen for the urothelium of embryonic and adult bladder (Fig. 2c) and for ureter. Again, an additional reaction in some suprabasal, and occasionally in intermediate cell bodies, was seen in renal pelvis (Fig.

4a).

The keratins K15, K2, K9 or the keratin pair K1/10 could not be detected in any of the urothelial tissues tested. Positive controls had been performed in human epidermis previously.

Detection of uroplakin

Uroplakin III was found at the apical surface of all cells facing the lumen of adult and embryonic bladder and in ureter. The immmunoreactions were seen forming

Fig. 1. Schematic summary of the keratin expression in different urothelial tissues, as revealed by immunostaining of cryosections. A weak immunoreactivity is shown as light pink, a clearly detectable immunoreactivity as orange, an intense immunostaining as deep red coloration of the cells. No reaction is demonstrated by unstained white cells (for details, e.g. antibodies used, see section material and methods).

a continuous band at the surface of the adult and embryonic bladder, as well as of the ureter, and were not detectable in the renal pelvis (data not shown).

Electron microscopy of superficial cells of adult bladder

Our electron microscopical examinations showed that the luminal plasma membrane of most superficial cells displayed scalloped formations, which were characterized by an asymmetric unit membrane (Fig. 5a,g). Higher magnification revealed that the outer membrane leaflet of these formations was prominently thicker than the inner leaflet (Fig. 5d-e) and represented the packed uroplakin particles associated with the outer

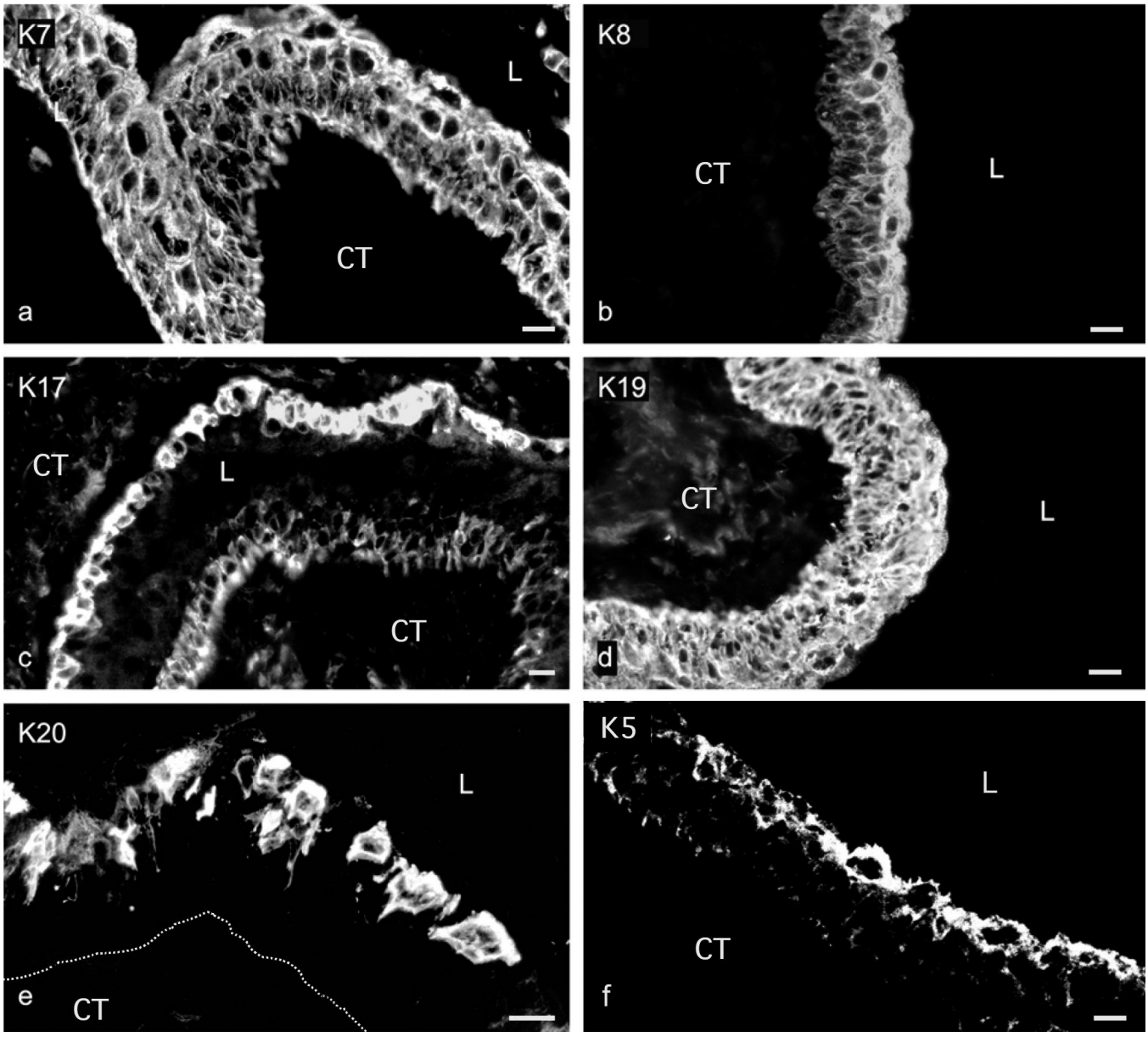

Fig. 2. Immunostaining of human bladder urothelium with antibodies against keratins K7, K8, K17, K19, K20 and K5. **a.** Antibodies against keratin 7 (RCK 105) stain all epithelial cells, with an accentuation of the luminal cells. **b.** A similar staining pattern is revealed with K8 antibodies with a stronger reaction intensity of the luminal cells (Ks17.2). **c**. Antibodies against keratin 17 (Ks17E3) react only with basal cells. The image shows a section of a folded bladder urothelium with two facing urothelial lines and a centred bladder lumen (L). **d**. A homogenous staining of all cells is obtained using antibodies against keratin 19 (Z105.6). **e.** Keratin 20 (IT Ks20.8) was restricted to most, but not all, cells facing the bladder lumen. Some cells show basally directed cellular extensions which do not reach the basal lamina. **f**. Most intense staining with keratin 5 antibodies (CK5.2) is observed in all luminal cells; reactions are less intense in basal cells, while intermediate cells show only weak or no reactions. L, bladder lumen; CT, connective tissue; dotted line in 2e, basal lamina. Scale bars: 20 µm.

leaflet of these special membrane areas (see e.g. (Sun, 2006)). These cells showed, in addition, densely organized apically located intermediate filaments with numerous, often flat vesicles. These vesicles were grouped or stacked together and had identical membrane asymmetries to the scalloped plasma membrane areas, and by this could be characterized as the uroplakin delivering s.c. fusiform or discoid vesicles (Fig. 5c,f). Characteristically, these cells also showed well developed dictyosomes and large numbers of lysosomes with dense granular and vesicular content.

While these superficial cells clearly showed all morphological criteria of typical umbrella cells (see also e.g. (Romih et al., 2005)), some of these luminal cells, however, lacked such features completely (Fig. 5b,g). Their apical plasma membrane showed no bulges and had a normal looking unit membrane without packed uroplakin particles. There was no obvious accumulation of apically located vesicles and the organization of the cytoskeletal elements was less dense. At the apical surface of this different type of superficial cell microvilli and coated pits (Fig. 5h,j) were more frequent than on the umbrella cells and their general morphology was comparable with the morphology of the intermediate and basal cells.

Discussion

Our investigations of adult and embryonic bladder, ureter and renal pelvis demonstrate that with the exception of the keratins K6 and K20 these tissues are characterized by a similar keratin pattern. Variations are mainly due to the distribution and amount of the various synthesized keratins within the epithelial layer.

The analysis of the simple keratins K7, 8, 18, 19 and 20 basically confirm earlier data obtained from adult urinary bladder, ureter and renal pelvis (Moll et al., 1988, 1990). Differences were seen in the extent of immunostaining of basal and intermediate cells for K8 and 18. While in our studies all cells reacted positive for these keratins throughout the epithelial layer (see for K8 also (Troyanovsky et al., 1989)), Moll and co-workers (Moll et al., 1988), using a different set of antibodies, reported only on a moderate reactivity of basal and

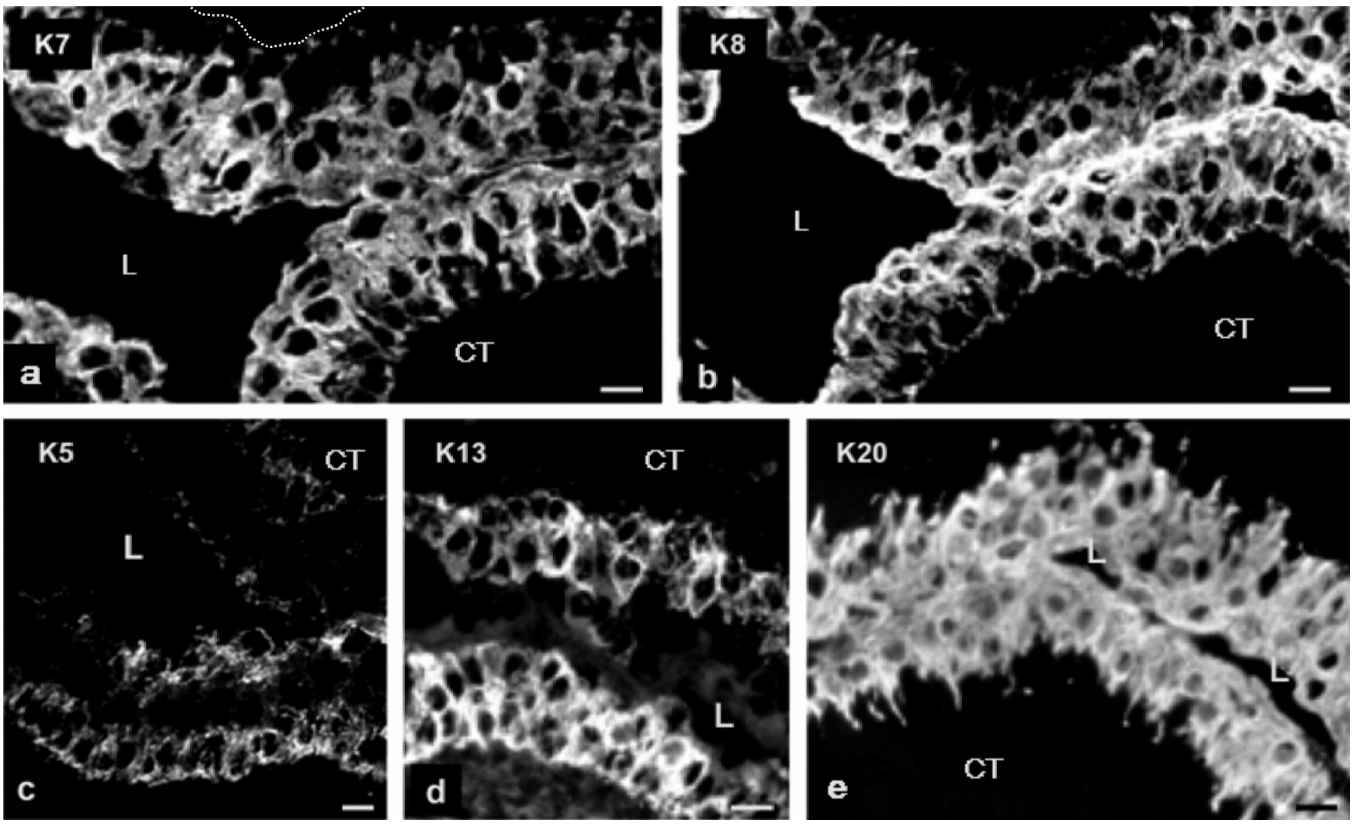

Fig. 3. Immunostaining of human embryonic bladder urothelium with antibodies against keratins K7, K8, K5, K13 and K20. **a**. Antibodies against keratin 7 (RCK 105) stain all suprabasal cells. Basal cells are always negative. **b**. A similar staining pattern is detected with antibodies against keratin 8 (Ks17.2). **c**. Keratin 5 antibodies (CK5.2) react with all luminal and basal cells while intermediate cells are barely stained. **d.** Antibodies against keratin 13 (2D7), stain all basal and the majority of intermediate cells. With a few exceptions superficial cells are always negative. **e.** Keratin 20 (antibody IT Ks20.8) is detected in all luminal and most suprabasal cells. L in a-e, bladder lumen; CT, connective tissue; dotted line in 3a, basal lamina. Scale bars: 20 µm.

intermediate cells.

The strong immunostaining of the above mentioned simple keratin types K8, K18, K19 and of K20 pointed to a dense organisation of keratins in these cells, which could be confirmed by electron microscopy. Especially in subapical areas of umbrella cells (see below) a dense network of intermediate filaments was seen and represented part of the so-called crusta described by light microscopy (Teutsch, 1977). Such specific organisation of the intermediate filaments within this subluminal region was also described by Veranic and Jezernik (2002) and is different from the more loose arrangement of filaments in the basal and intermediate located cell bodies. According to these electron microscopical observations we do not think that a cell-type specific epitope masking in basal and intermediate cells might be responsible for the reduced staining intensity of the above mentioned keratin isotypes, as has been proposed by Moll et al. (1988). With antibodies against Keratin 20, which has also become a marker protein of urothelial tissue since its first description (Moll et al., 1990) we regularly found in adult bladder some K20 negative superficial cells, indicating a heterogeneity of the cells facing the bladder lumen. A similar observation has been described by Riedel and coworkers (Riedel et al., 2005).

Such heterogeneity matched well with our electron microscopical data, where we found surface cells with all characteristic features of well differentiated umbrella cells next to a subpopulation of cells with a different morphology, and which obviously represented not yet fully differentiated umbrella cells. Similar observations were reported by Veranic et al. (2004) using regenerating murine bladder as a model system to describe urothelial differentiation. We conclude from this that although both cell types reacted positive for uroplakin III by immunofluorescense microscopy, maturation to umbrella

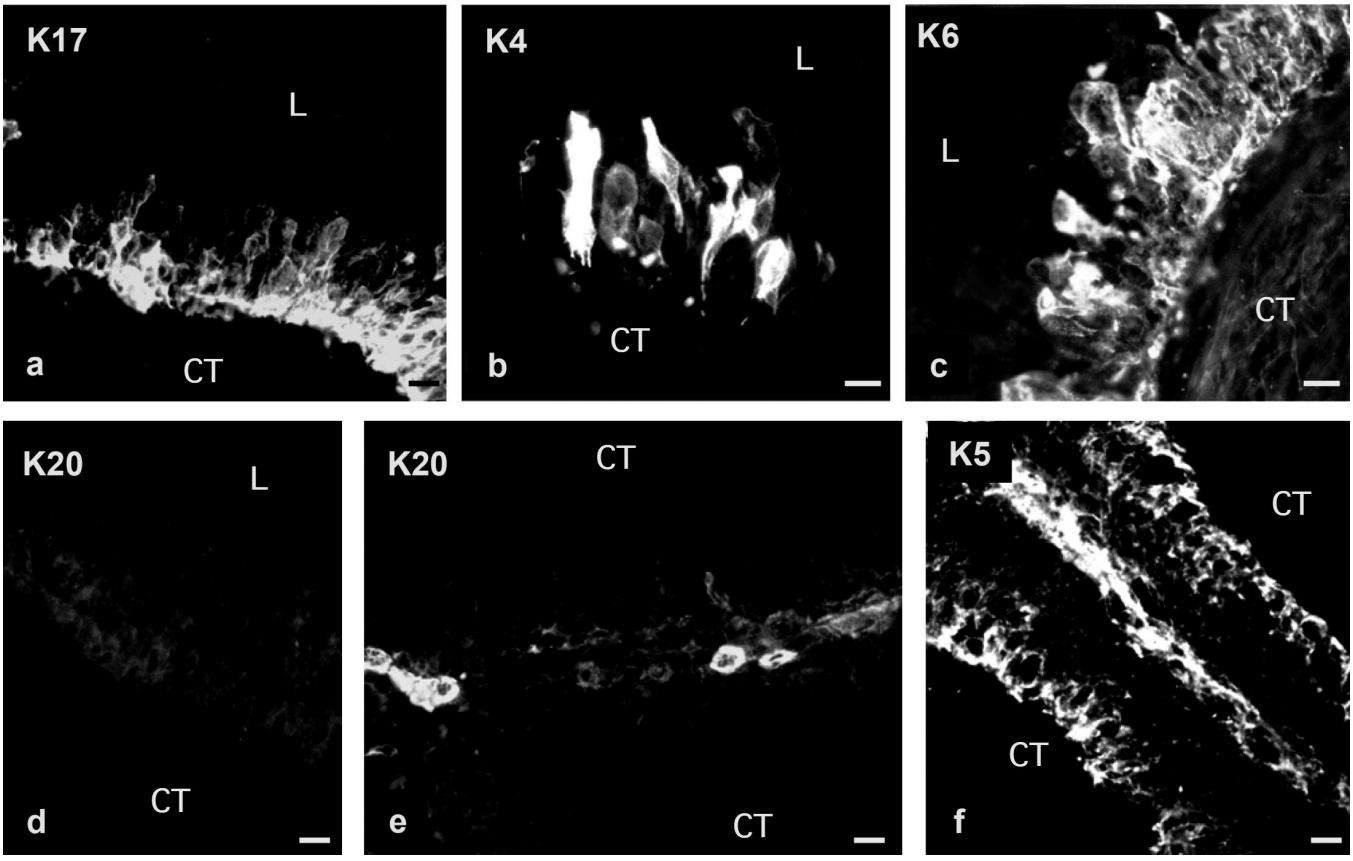

Fig. 4. Immunostaining of the urothelium of the renal pelvis **(a-d)** and the ureter **(e-f)** with antibodies against K17, K4, K6, K5 and K20. **a.** In renal pelvis Keratin 17 (Ks17E3) is found in all basal cells, additionally in some suprabasal and occasionally in intermediate cells. **b.** Keratin 4 (6B10) is detected in some basal and intermediate cells, while superficial cells show no immunoreactivity in renal pelvis. From all tested tissues only the urothelium of the renal pelvis **c.** reacts positive with antibodies against Keratin 6 (KA12). The reactions are restricted to basal and suprabasal cells and to a small number of intermediate cells. **d.** No immunoreactivity is observed in the urothelium of the renal pelvis using a variety of antibodies against keratin 20. **e.** In the urothelium of the ureter some superficial cells stain positive for keratin 20 (IT Ks20.8). **f.** All basal and luminal cells of the ureter are keratin 5 (CK5.2) positive, while suprabasal and intermediate cells are only poorly stained. Note that in figure f the ureter lumen is collapsed, causing a direct contact of the luminal surfaces of the superficial cells. L, bladder lumen; CT, connective tissue. Scale bars: 20 µm.

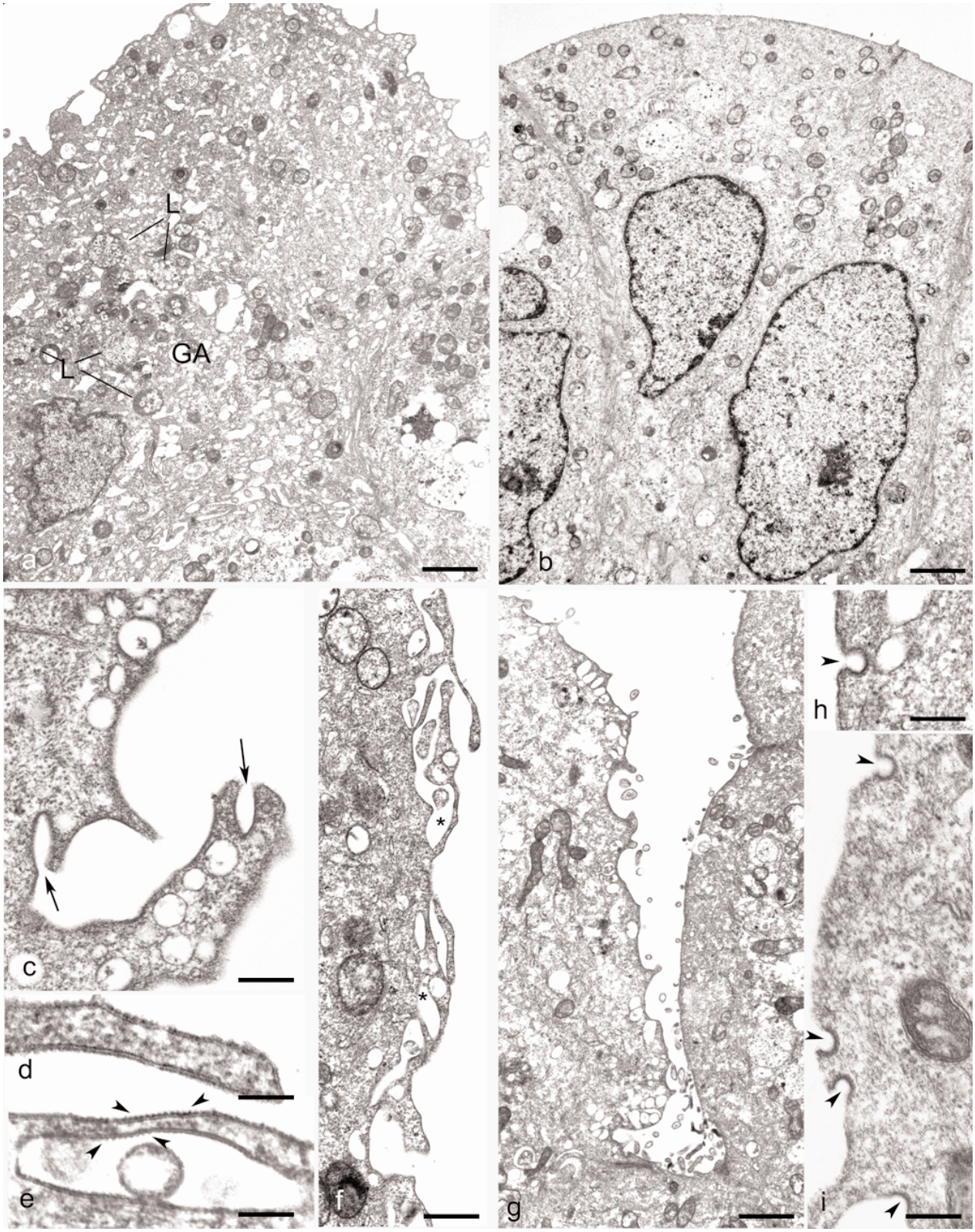

Fig. 5. Electron microscopy of superficial cells of adult bladder. The survey pictures (**a, b, g**) demonstrate the two different luminal cells types. Type I illustrates the typical appearance of umbrella cells with a scalloped surface, and numerous vesicles in the cell periphery **(a and left cell in g)**. Type II cells **(b and right cell in g)** are characterized by a smooth, continuous surface and no obvious vesicle formations underneath the plasma membrane. Higher magnification of typical umbrella cells **(c-f)** revealed characteristic features like uroplakin delivering s.c. fusiform or discoid vesicles **(arrows in c, and e.g. asterisk in f)**, the asymmetric plasma membrane with the outer membrane leaflet prominently thicker than the inner leaflet (**d, e**; prominent formations of uroplakin particles are marked with arrowheads) and the apically located dense cytoskeletal network **(c, f)**. The smooth surfaced type II cells **(b, right cell in g, h, j)** did not show any of these features but often the formation of coated pits could be found at the cell surface **(arrowheads in h and j)**. L, lysosomes; GA, Golgi area. Scale bars: a, b, g, 4 µm; c, f, i, 1 µm; d, e, 0.5 µm.

cells needs the synthesis of the uroplakins (i), the organization of uroplakin plaques in the form of microridges leading to the appearance of an asymmetric plasma membrane (Wu et al., 1994) with scalloped membrane formations and discoid vesicles (ii), and the synthesis of K20 (iii). The existence of immature umbrella cells in a luminal position further supports the hypothesis of Moll et al. (1988) and the observation of de la Rosette et al. (2002) that umbrella cells develop from cells in the underlying intermediate epithelial location. This hypothesis is further supported by our description of K20 positive long cytoplasmic cellular tails reaching deep into regions of intermediate cell bodies, yet with lost basal contact. These observations might allow to speculate that maturation to umbrella cells might be triggered by the loss of adhesion to elements of the basal lamina and by signals upon contact with urine fluid.

The observation of K20 positive cells with lost contact to basal membrane provide further evidence for the unique role of umbrella cells and reveal characteristics of a stratification for this urothelial layer.

However, this interpretation does not hold for embryonic bladder with a much broader K20 synthesis, also in most intermediate cells, an expression pattern which closely reflected situations described for urothelial dysplasia (Mallofre et al., 2003; Retz et al., 2003; Kunju et al., 2005). At present we do not know if mature umbrella cells exist in embryonic bladder but we do not believe that the impermeability of transitional epithelium is directly related to K20 synthesis as discussed by de la Rosette (de la Rosette et al., 2002) or depends on the formation of the uroplakin plaques (Hu et al., 2001, 2002). We rather believe that impermeability in adult, as well in embryonic bladder epithelium, is dependent on the development of an intact continuum of tight junctions which we could identify in all tested urothelial organs associated with cells lining the luminal surfaces (Langbein et al., 2002a) (see also (Acharya et al., 2004) for mouse and rat bladder). This might also hold for the superficial cells of the ureter with dominantly K20 negative superficial cells and especially for the K20 negative regions of the renal pelvis.

It has been discussed that the general elasticity of the umbrella cells is dependent on the presence of K20 (Vaidyanathan et al., 2002; Veranic et al., 2004). A comparison of our data showing high amounts of K20 in bladder, but lower amounts in the ureter and a K20 negative renal pelvis supports this theory. An increasing volume strain and the adaptation to increasing volume changes from renal pelvis to the bladder might influence K20 expression. However, the missing K20 reactions might also be explained by the differing pathways in embryogenesis. Following the theory of urothelial origin during embryogenesis (for a review see (Sun, 2006)), human pelvis epithelium derives from the mesoderm, in contrast to the bladder urothelium, which develops from the endoderm, like e.g. the K20 positive intestinal tract.

While the K5 expression in basal and intermediate cells is roughly what had been described in previous studies, with either small amounts of K5 in all cells of the epithelial layer (Moll et al., 1988), or with an intense staining in basal cells and a restricted reactivity in intermediate cells (de la Rosette et al., 2002), or with the restriction of K5 only to basal cells (Southgate and Harnden, 1999), the strong expression of K5 in superficial cells seen in embryonic and adult bladder and in the ureter, and to a lesser extent in renal pelvis, is surprising, and has not been described before. As the results of the above mentioned authors were all obtained from paraffin sections an incomplete unmasking cannot be excluded, and might be the reason for these controversial findings.

The poor and mostly restricted expression to the basal cells of the K5 potential partner protein K14 with an indiscernible filamentous form apparently argues against the presence of K14 containing filaments in urothelial tissue, especially as similar difficulties to demonstrate K14 were also reported by de la Rosette et al. (2002). The synthesis of K14 protein, however, was confirmed by our immunoblotting reactions (results not shown). The presence of keratin 14 in basal cells has also been documented by Kurzrock et al. (2008) immunohistochemically; however, keratin 14 expression was not limited to the so-called “label-retaining cells” in urothelium which have been identified as tissue stem cells in skin, cornea, intestine and prostate. The lack of any staining reactivity with K14 antibodies in intermediate and especially in K5 positive superficial cells indicated that type I K5 in these cells must be associated with one of the type II simple epithelia keratins.

While K5 and K14 are otherwise found in basal cells of pseudostratified and of non-cornified and cornified epithelia, the keratin pair K4/K13 is a characteristic component of suprabasal cells of non-cornified squamous epithelia. In the various urothelia tested all basal and all intermediate cells, with some reduction in renal pelvis, were positive for K13, as has been seen by other authors (Moll et al., 1988; Schaafsma et al., 1989). In contrast to de la Rosette et al. (2002) who found intense immunstaining throughout the full thickness of the urothelium of embryonic bladder we here detected only occasionally K13 positive superficial cells. Since in adult urothelia K13-positive superficial (or umbrella) cells have never been described it can be concluded that the reduction of K13 synthesis in cells lining the luminal surface is a further step during maturation to umbrella cells. The potential keratin partner K4 appeared only scattered in basal and intermediate cells of bladder and renal pelvis and was not clearly detected in the ureter, similar to findings of other authors (Achtstatter et al., 1985; Moll et al., 1988; Schaafsma et al., 1989). Most, albeit also scattered, K4 positive cells were also found in embryonic bladder and also included some superficial cells. This synthesis of K4 in embryonic bladder has not yet been described.

The scattered staining of intermediate cells of

various locations within the epithelial layer with antibodies against K4 allowed the tracing of basally oriented cellular extension, even of the uppermost intermediate cells down to the level of the basal lamina. This finding clearly documents that this part of the urothelium represents the typical morphological characteristics of a pseudostratified epithelium.

The positive reactions with antibodies to K17 in basal cells of the urothelia tested represented the staining pattern known from members of the group of "complex epithelia" (pseudostratified-, transitional-, myo-epithelia) which express keratins of stratified, as well of simple epithelia (Franke et al., 1980; Moll et al., 1982; Cooper et al., 1985), and K17 can be considered as the characteristic and specific keratin of this group (Troyanovsky et al., 1989). Keratin 17 in basal cells of normal bladder was first described by Troyanovsky and co-workers (Troyanovsky et al., 1989), a result which we can confirm (see also (Laguna et al., 2006)) and complete with new data on embryonic bladder, ureter and renal pelvis. Our finding of K17 positive cells in embryonic bladder is in contrast to results of de la Rosette et al. (de la Rosette et al., 2002), who, using the same antibody (E3), but working with paraffin sections, did not see any immunoreaction in 38 of 40 foetuses. The numerous positive suprabasal cells found in renal pelvis is indicative of an increased K17 synthesis, differed from the staining pattern of the other urothelial tissues. A similar upregulation of K17 was described for regenerating rat urothelium and was correlated with undifferentiated cells (Romih et al., 2002), and a rapidly induced K17 was described for wounded stratified epithelia (Kim et al., 2006).

Similarly, the detection of K6 in basal and suprabasal cells of the pelvic urothelium is particular and might also be explained by different pathways in embryogenesis, causing a differing keratin expression in pelvis and the other urothelia tested. Generally, expression of K6 and its isoforms have been described for a variety of different and not related tissues e.g. for normal cervical epithelium (Smedts et al., 1993), hair follicle (Rothnagel et al., 1999; Langbein et al., 2002b), eccrine sweat glands (Langbein et al., 2005), and has been related with activated epidermis during wound healing or certain skin diseases (Rothnagel et al., 1999; Mommers et al., 2000; Komine et al., 2001; Wong and Coulombe, 2003), and associated with early stages of mammary gland development (Grimm et al., 2006). In vitro, keratin 6 expression in a human urothelial cell line has been suggested to be a biomarker for malignant urothelial transformation (Somji et al., 2008).

The K6 and the above mentioned expanded K17 expression might indicate morphological and functional differences of the renal pelvis in comparison with the other urothelial tissues. Such differences would be in line with the theory of differing urothelial origin during embryogenesis, as discussed above for K20. Common for both keratins is that their expression is broadly correlated with a proliferate activity of a tissue (Rothnagel et al., 1999; Mommers et al., 2000; Wong and Coulombe, 2003). A direct relation to a proliferative activity to the epithelium of the renal pelvis, however, is not understood/clear.

The epithelia of the various tested human urothelial tissues show the combined synthesis of keratins that are otherwise characteristic for non-cornified squamous, for pseudostratified and for monolayered/simple epithelia, including K20, which is otherwise restricted to gastric and intestinal epithelium and to Merkel cells. The classification of the urothelium either to represent a stratified (Moll et al., 1988; Jost et al., 1989; Sun, 2006) or a pseudostratified epithelium (Bulger, 1988) is controversially discussed. Our demonstration of fluorescent labeled cellular extension contacting the basal lamina, with the exception of the cells facing the bladder lumen, demonstrates that the intermediate cells, representing the major part ot the urothelium, clearly resemble the morphological criteria of a pseudostratied epithelium.

Moll and coworker (1988) consider the presence of the "stratification related cytokeratin 13" to classify the urothelium as a stratified epithelium as did Jost et al. (Jost et al., 1989) using the argument that the surface cells, which do not reach the basal lamina, form a stratification-like cell layer on top of the other cells. This stratification-mimiking cell layer, however, is negative for K13 and expresses, with the exception of K5, only keratins present in simple/mono-layered epithelia.

The morphological organisation of the adult human urothelium among the epithelia is as unique as is its molecular composition, with a set of ten different keratins, and can neither be correctly classified to represent a pseudostratified nor a stratified epithelium. Differences might exist for the embryonic urothelia and areas of the renal pelvis with less differentiated or even missing umbrella cells (see also (Romih et al., 2005)). Despite the mixture of keratin expression profiles of pseudostratified, non cornified stratified and simple epithelia, the urothelium can be identified molecularly by the combined detection of K13 and the simple epithelia keratin types, especially K20.

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