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Uroplakins, Specific Membrane Proteins of Urothelial Umbrella Cells, as Histological Markers of Metastatic Transitional Cell Carcinomas

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Uroplakins (UPs) Ia, Ib, II, and III, transmembrane proteins constituting the asymmetrical unit membrane of urothelial umbrella cells, are the first specific urothelial differentiation markers described. We investigated the presence and localization patterns of UPs in various human carcinomas by applying immunohistochemistry (avidin-biotin-peroxidase complex method), using rabbit antibodies against UPs II and III, to paraffin sections. Positive reactions for UP III (sometimes very focal) were noted in 14 of the 16 papillary noninvasive transitional cell carcinomas (TCCs) (88%), 29 of the 55 invasive TCCs (53%), and 23 of the 35 TCC metastases (66%). Different localization patterns of UPs could be distinguished, including superficial membrane staining like that found in normal umbrella cells (in papillary carcinoma), luminal (microluminal) membrane staining (in papillary and invasive carcinoma), and, against expectations, peripheral membrane staining (in invasive carcinoma). Non-TCC carcinomas of various origins (n = 177) were consistently negative for UPs. The presence of UPs in metastatic TCCs represents a prime example of even advanced tumor progression being compatible with the (focal) expression of highly specialized differentiation repertoires. Although of only medium-grade sensitivity, UPs do seem to be highly specific urothelial lineage markers, thus opening up interesting histodiagnostic possibilities in cases of carcinoma metastases of uncertain origin. (Am J Pathol 1995, 147:1383–1397)

In the histopathological diagnostic procedures currently applied to the patients presenting with carcinoma metastases, the location of whose primary tumor is uncertain or unknown, histological differentiation markers continue to be of increasing importance.[1,2] Unfortunately, markers that are specific for a single epithelium or organ are available only for a few types of carcinoma, eg, prostate-specific antigen and thyroglobulin for prostate and thyroid carcinomas, respectively. Cytokeratin polypeptide typing of carcinomas may provide valuable pointers concerning the origin of metastases, but only rarely are these patterns sufficient to definitely identify the primary organ with any certainty.[3]

Transitional cell carcinomas of the urinary tract, when invasive, also may give rise to lymph node and distant metastases, and in such cases, diagnosis can be a problem. Although many studies have examined possible tumor markers of transitional cell carcinomas by applying a variety of antibodies with greatly differing specificities, the markers described have been associated either with malignant transformation[4–6] or with tumor progression,[7] or their expression has been found to show some correlation with the degree of malignancy and the prognosis, eg, epithelial membrane antigen[8,9] or oncogene/tumor suppressor gene products.[10–12] Meanwhile, other markers that have been investigated are mainly

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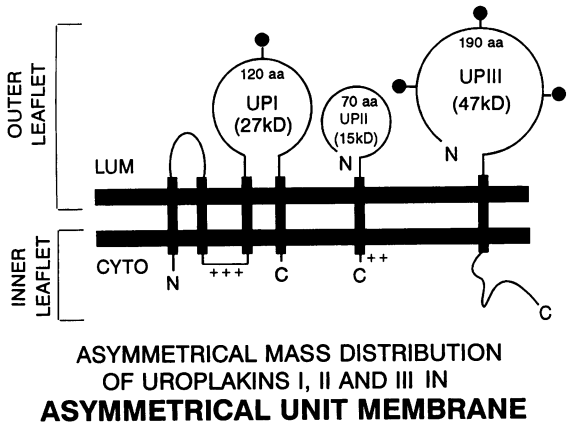

Figure 1. *Schematic representation of the molecular organization of uroplakins UP Ia, UP II, and UP III in relation to the plasma membrane. The asymmetrical mass distribution explains the asymmetry of the unit membrane in the urothelial plaque.*

expressed in superficial bladder tumors but seem to disappear in invasive and metastatic transitional cell carcinomas.[7,13] Nearly all of the previously described antibodies also stain, to a greater or lesser degree, nonurinary epithelia and carcinomas. Certain antigens, such as involucrin,[14] E48 antigen,[15,16] and SCC antigen,[17] are markers shared by both transitional and stratified squamous epithelia and their carcinomas. As yet, no differentiation (or lineage) marker specific for transitional cell carcinomas and their metastases has been described.

Normal urothelium, however, does contain tissue-specific differentiation products that have been well characterized both morphologically and, more recently, biochemically, ie, urothelial plaques (diameter, 0.1 to 0.5 μm), large numbers of which are present in the superficial plasma membrane of urothelial superficial (umbrella) cells. These plaques, which can already be detected in cytoplasmic vesicles, are characterized by a highly unusual membrane structure, ie, the asymmetric unit membrane (AUM), the luminal leaflet of which is twice as thick (8 nm) as its cytoplasmic leaflet (4 nm).[18,19] The thickening of the luminal leaflet is a result of the presence of particles (diameter, 12 nm) exhibiting a semicrystalline organization, the molecular composition of which has recently been demonstrated.[20–22] The molecular constituents principally comprise four transmembrane proteins, ie, uroplakin (UP) Ia (27 kd), UP Ib (28 kd), UP II (15 kd), and UP III (47 kd; the size of the core protein is 28.9 kd). These UPs, particularly UP Ia, UP Ib,[23] and UP II,[24] are characterized by their markedly asymmetric mass distribution, with the extracellular domain being considerably larger than the intracellular one (Figure 1). This may account for the clearly visible ultrastructural thickening of the luminal leaflet of the unit membrane. UP III probably plays a role in the formation of the urothelial glycocalyx and may interact, via its cytoplasmic portion, with the cytoskeleton (possibly with cytokeratin filaments).[25]

In the present study, we used antibodies against UPs to perform a broad-based immunohistochemical screening of primary and metastatic transitional cell carcinomas as well as other carcinoma types and corresponding normal tissues, the aim being to determine whether UPs might serve as histological markers of human transitional cell carcinomas and their metastases.

Materials and Methods

Tissues

We used paraffin blocks of various normal and malignant human tissues stored in the Institute of Pathology, Mainz. The tumors included 106 transitional cell carcinomas (6 of the ureter, 9 of the renal pelvis, the remaining ones of the urinary bladder); of these, 71 were primary tumors, 31 were lymph node metastases, and 4 were distant metastases (in 8 cases, primary tumor and metastasis tissues from the same patient were used). In addition, we examined paraffin-embedded tissue blocks of 177 primary and metastatic carcinomas derived from other organs. Also, a large spectrum of normal adult tissues (present in tumor blocks or in separate blocks; in most cases, specimens from several patients per tissue type) as well as normal tissue obtained during fetal autopsies were included in the study. For antibody testing, fresh snap-frozen tissue from normal human ureters (obtained in the course of frozen section diagnosis) was used.

Antibodies

The following antibodies against UPs were used.

Anti-UP III

AUMs were isolated from bovine urinary bladders (some fresh, some frozen batches, with similar results) by a sucrose density gradient followed by a selective detergent wash.[21] The AUM fraction was then used to immunize rabbits. Antisera from two different animals (A and B) were used.

When subjected to Western blotting, antiserum A exhibited a specific reaction only with UP III. With paraffin sections, antiserum A (dilution, 1:2000) revealed the typical UP staining pattern of the urothelium (see

Results). A diffuse background staining by this antiserum for smooth muscle cells and collagen fibers was eliminated by preabsorbing it against a collagen-fiber-rich tissue preparation (pooled, deparaffinized, rehydrated, and trypsinized paraffin sections of human breast tissue with fibrocystic disease). Specifically, the antiserum was diluted (1:100) in phosphate-buffered saline (PBS) containing 1% bovine serum albumin and shaken with an appropriate amount of the tissue preparation at room temperature for 20 minutes, and the absorbent material was removed by centrifugation (10 minutes at 13,000 × *g*). (This preabsorption procedure was repeated six to eight times). The purified antiserum was designated anti-UP III(A) and was used at a final dilution of 1:500.

Antiserum B also demonstrated strong Western blot reactivity with UP III but in addition showed moderate reactivity with UP Ib and weak reactivity with UP II.[21,22] This antiserum was preabsorbed against a tissue preparation of tunica muscularis propria of human stomach (prepared from paraffin sections as described above). This purified antibody preparation was designated anti-UP III(B) and was used at a final dilution of 1:2000.

During the extended screening of paraffin sections described in Results, occasional cross-reactivities with certain epithelial cells were observed, which, however, were different for the two antibody preparations. Thus, antibody anti-UP III(A) produced staining of certain mucous materials (such as the mucus vacuoles in some gastric pyloric gland cells as well as in scattered signet ring cells of some stomach and some invasive lobular breast carcinomas) as well as fuzzy staining of luminal membranes in certain adenocarcinomas, particularly those of the colon. This cross-reactivity was not observed with anti-UP III(B). On the other hand, anti-UP III(B) exhibited cross-reactivity with the luminal surface of some serous ovarian carcinoma cells, with some pancreas carcinoma cells and, occasionally, with normal intrahepatic bile ducts. No such cross-reactivity was observed with anti-UP III(A). Because of their non-overlapping, these cross-reactivities were considered not to be UP III specific (see Discussion). Rather, in the present study on paraffin sections, the specimens were recorded as UP III positive only when both antibodies (anti-UP III(A) and antibody UP III(B)) produced similar staining on serial sections (cf Figure 3, b and c).

Anti-UP II

A peptide (ELVSVVDSGSG) corresponding to amino acid residues 1 to 11 of mature bovine UP II was chemically synthesized, cross-linked to keyhole limpet hemocyanin, and used to immunize the rabbit.[22] The resulting antiserum was affinity purified with the above peptide coupled to Affi-Gel 15 (Bio-Rad, Hercules, NY).

Anti-UP Ic

Monoclonal mouse antibody AE31 against UP Ic[20,23] was used as an undiluted cell culture supernatant.

Immunohistochemistry

For initial antibody testing, 5-μm-thick cryostat sections of unfixed, snap-frozen human ureter tissue were cut, air dried, fixed in acetone (15 minutes at −20°C) and then air dried again. Indirect immunoperoxidase microscopy was performed by using a standard protocol.[26] As secondary antibodies, peroxidase-conjugated swine antibodies against rabbit immunoglobulins or peroxidase-conjugated rabbit antibodies against mouse immunoglobulins (Dako, Hamburg, Germany) were applied. The staining reaction was performed with 3,3′-diaminobenzidine and H_2O_2.

For the staining of paraffin sections, the avidin-biotin-peroxidase complex (ABC) method[27] (ABC kit; Vector Laboratories, Burlingame, CA) was applied. Approximately 4-μm-thick paraffin sections were dried overnight at 60°C and, after deparaffinization and rehydration, endogenous peroxidase activity was blocked by incubation with 0.6% H_2O_2 and 40% methanol in PBS for 30 minutes. Subsequently, the sections were treated with 0.1% trypsin (Sigma, Munich, Germany) in 0.05 mol/L Tris-HCl (pH 7.8) at 37°C for 15 minutes. After incubation with normal goat serum (diluted 1:10 in PBS), the sections were incubated overnight at 4°C with an appropriately diluted (see above) rabbit antibody. Bound antibody was detected by using the ABC kit (containing biotinylated goat antibodies against rabbit immunoglobulins). The staining reaction again involved the application of a diaminobenzidine solution containing H_2O_2. Slides were weakly counterstained with Mayer's hematoxylin (Merck, Darmstadt, Germany). As negative controls, the specific primary antibody was replaced by a nonrelevant rabbit antibody. These controls consistently yielded negative results. In each staining run, a positive control section (human renal pelvis tissue with a transitional cell carcinoma or human ureter tissue) was included.

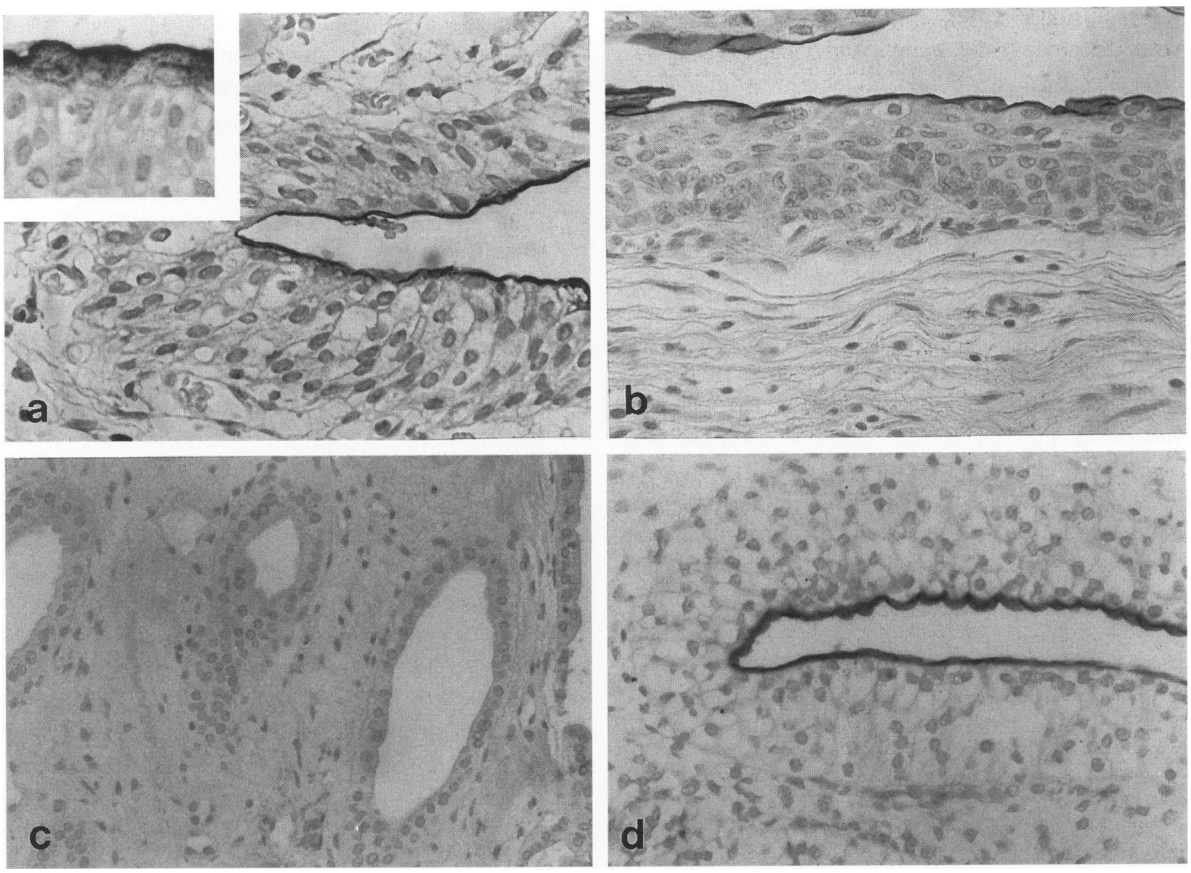

Figure 2. *Immunohistochemical detection of uroplakins in normal adult and fetal human tissues.* **a** *and* **b**: *UP III (anti-UP III (A))* (**a**) *and UP II* (**b**) *in adult urothelium of the ureter* (**a**), *the bladder* (**inset** *in* **a**), *and the renal pelvis* (**b**), *being specifically localized in the apical membrane of the umbrella cells. An example of additional, finely granular cytoplasmic staining is shown in the* **inset** *in* **a**. **c**: *No staining for UP III (anti-UP III (A)) is seen in the columnar surface lining epithelium (right) and the collecting ducts of adult renal papilla.* **d**: *Developing renal pelvis urothelium (13-week-old fetus), already showing the typical apical membrane reaction of the superficial cells for UP III (anti-UP III (A)). Magnifications:* **a**, ×*380;* **inset**, ×*360;* **b**, ×*280;* **c**, ×*180;* **d**, ×*240.*

Results

Normal Human Tissues

To assess the reactivities of our antibodies to bovine UPs with human UPs, we applied them to frozen sections of human ureter tissue using the indirect immunoperoxidase method. All three rabbit antibodies (anti-UP III(A), anti-UP III(B), and anti-UP II) as well as monoclonal antibody AE31 specifically stained the superficial cell membrane of the umbrella cells of the urothelium (not shown).

On paraffin sections of human tissue routinely fixed in formalin, only the rabbit antibodies produced a similar specific reaction for normal urothelium (Figure 2, a and b), this being, in fact, even stronger than that observed in frozen sections; in contrast, antibody AE31 failed to react at all, even though several immunohistochemical staining methods were tried, including microwave oven pretreatment.[28] The staining of the normal urothelium produced with the antibodies against UP III (Figure 2a) and UP II (Figure 2b) was always confined to the superficial umbrella cells, so that it generally had the appearance of a markedly linear and, in well preserved areas, continuous decoration of the superficial (apical) cell membrane. In some cases, in addition to the strong apical membrane staining, weaker, finely granular cytoplasmic reactivity, probably corresponding to AUM vesicles, was observed in umbrella cells (Figure 2a, inset).

The most extensive tissue screening was performed with the anti-UP III antibodies. Similar UP III staining was recorded in urothelium of all sites, including renal pelvis, ureter, urinary bladder, and prostatic urethra. In cases of urocystitis cystica, luminal membrane reactivity for UP III was observed in some but not all of the microcystic structures. A negative reaction for UP III was invariably recorded for all other human tissues, including skin, mammary gland, oral mucosa, salivary glands, paranasal sinus

Table 1. *Uroplakin III in Human Carcinomas**

	No. of cases	No. of positive cases	UP III pattern†							
			Superficial		Luminal		Peripheral		Others‡	
			+	(+)	+	(+)	+	(+)	+	(+)
Transitional cell carcinomas of the urinary tract										
Papillary noninvasive (Ta)§	16	14 (88%)	7	7	9	4	0	1	1	1
Invasive (T1–T4)	55	29 (53%)			10	16	11	9	0	5
Metastases (N, M)‖	35	23 (66%)			6	8	11	7	1	3
Carcinomas of other organs¶	177	0 (0%)								

*As determined immunohistochemically on paraffin sections.
†+, abundant or focal but still moderately numerous positive structures; (+), few or sparse positive structures. For some single tumors, several patterns were present and recorded (cf Table 2).
‡Intercellular, cytoplasmic.
§Including noninvasive portions of T1 tumors (6 cases) the invasive portions of which were not investigated.
‖Thirty-one lymph node metastases, four distant metastases; for localizations, see Table 2.
¶See text; 106 primary tumors, 71 metastases.

mucosa, lung, different parts of stomach, small and large intestine, rectoanal transitional zone, liver, bile ducts, pancreas, peritoneum, kidney parenchyma and surface epithelium of the renal papillae (Figure 2c), membranous and penile urethra, glans penis, prostate gland, ductus deferens, vagina, uterus, fallopian tube, ovary, adrenal glands, various mesenchymal tissues including blood vessels, muscle tissue, peripheral nerves, and placenta.

In fetal tissues, specific luminal UP III reactivity was detected in the developing urothelium of the renal pelvis and urinary bladder of both a 13-week-old (Figure 2d) and a 21-week-old fetus, whereas the various other fetal tissues tested, including kidney, liver, intestine, and skin, were entirely negative.

Carcinomas

The results of the immunohistochemical staining of carcinomas for UP III are summarized in Table 1.

Of the 106 transitional cell carcinomas studied, 16 were of the superficial papillary type and were either noninvasive (Ta) or contained major noninvasive portions (T1 cases). Of these 16 cases, 14 (88%) exhibited positive reactions for UP III. Several patterns of UP III immunostaining were observed in papillary tumor structures, the most frequently encountered of which was a linear *superficial* membrane reaction in the upper cell layer (Figure 3a), ie, staining of the cell membrane bordering at the lumen of the bladder, ureter, or renal pelvis. This staining was basically similar to the normal urothelial pattern but was more variable in intensity and usually discontinuous, with some cases exhibiting only very focal areas of staining. The other frequently observed pattern, usually occurring in combination with the first pattern, was a *luminal* (or microluminal) pattern, ie, membrane staining of more-or-less numerous, variably sized, morphologically often inconspicuous lumina within the papillary carcinomatous epithelium (frequently at a position relatively near to the surface; Figure 3a). Some cases exhibited an abundance of such UP-III-positive lumina (Figure 3, b–e). Some of the lumina contained amorphous material that demonstrated partial immunostaining (Figure 3, a, lower arrow, and d and e, left side). In some cases, the lumina, which were UP III positive (Figure 3e), were particularly conspicuous and comparably large, giving rise, in H&E-stained sections, to a morphological picture suggestive of glandular differentiation (Figure 3d). On very rare occasions, other reaction patterns (ie, *basal/peripheral*, *intercellular*, *cytoplasmic*; Table 1; Figure 3, f and g) were detected. Only 2 of these 16 cases (12%) were entirely negative.

Among the invasive transitional cell carcinomas (invasive portions; T1, n = 9; T2, n = 18; T3, n = 23; T4, n = 5; grades G2, G3, and G3 to G4) examined, positive reactions for UP III, ranging from sparse to abundant staining, were found in 29 of the 55 cases (53%; Table 1). Again, several staining patterns could be distinguished (Table 1), the most common of which was the *luminal* pattern, ie, membrane staining along variously sized lumina. A few cases with pseudoglandular morphology exhibited large and conspicuous debris-filled lumina (Figure 4a) showing specific UP III reactivity along their lining membranes (Figure 4b). Most cases, however, ie, the typical solid transitional cell carcinomas, contained scattered, mostly small and inconspicuous intercellular and/or intracytoplasmic lumina, which, surprisingly, were clearly outlined by the UP III antibodies (Figure 4, c and d). Although some cases contained a fairly large number of UP-III-positive lumina, careful screening revealed only very few of these in other tumors examined. UP-III-negative lumina were also observable in some cases. A second, less frequently

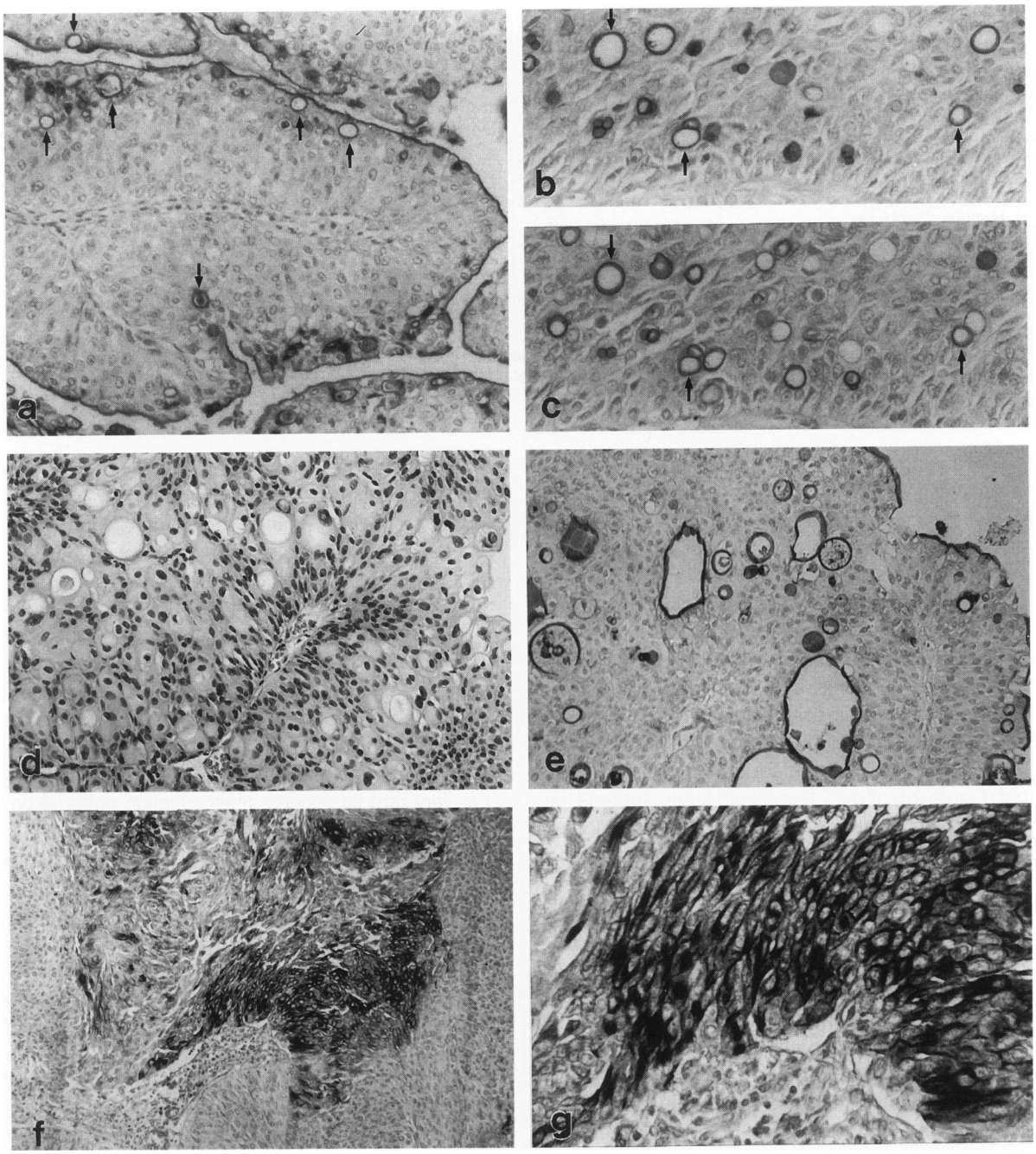

Figure 3. *UP III in papillary transitional cell carcinomas.* **a**: *G2 carcinoma of the bladder (pT1) showing linear apical membrane staining of the superficial cells (umbrella cell equivalents) and, in addition, membrane staining of small near-surface lumina* (arrows). **b** *and* **c**: *Renal pelvis tumor (G2; pTa) with particularly abundant small lumina, which show identical positive staining with both antibodies anti-UP III (A)* (**b**) *and anti-UP III (B)* (**c**) *as shown in consecutive serial sections (some corresponding lumina are denoted by* arrows). **d** *and* **e**: *Papillary bladder tumor (G1; pTa) with glandular differentiation reflected by the presence of multiple comparatively large lumina* (**d**, *H&E staining*) *that are strongly outlined by anti-UP III (A) antibodies* (**e**). **f** *and* **g**: *Papillary transitional cell carcinoma of renal pelvis (G2; pTa) with unusual, conspicuous cytoplasmic and intercellular membrane staining for UP III (anti-UP III (A)). Magnifications:* **a** *to* **c**, ×*180;* **d** *and* **e**, ×*140;* **f**, ×*80;* **g**, ×*280.*

encountered pattern (*peripheral* pattern) took the form of focal linear staining of the peripheral (seemingly basal) cell membrane of tumor cells bordering the stroma, this often being accompanied by the detachment of these cells from the stroma (Figure 4, e and f). This last feature was suggestive of genuine detachment of the tumor cell complexes in the sense of a pseudo-lumen formation; however, the possibility of this phenomenon being the product of a retraction artifact upon tissue fixation could not be

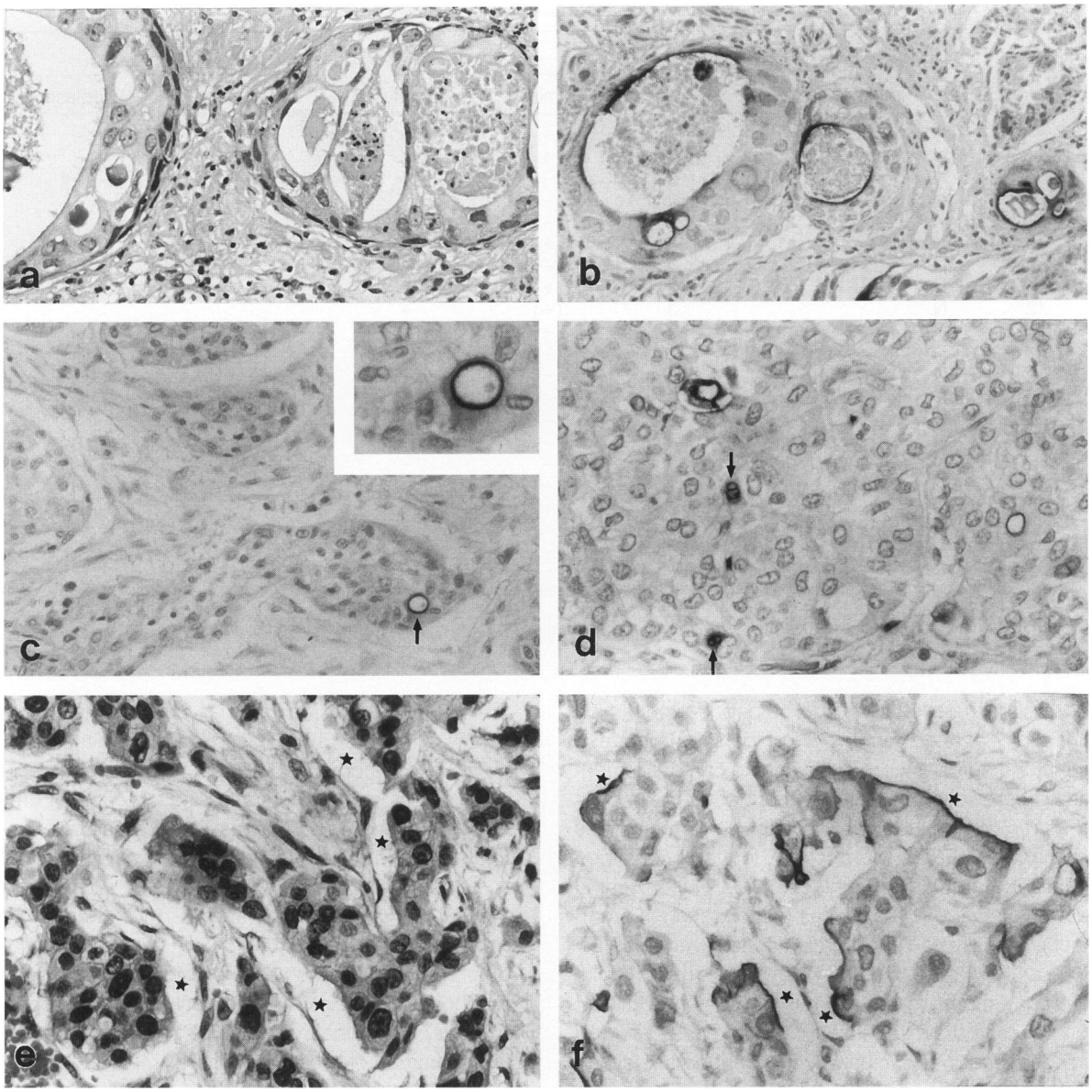

Figure 4. *Immunohistochemical detection and staining patterns of UP III (anti-UP III (A)) in invasive transitional cell carcinomas.* **a** *and* **b**: *Bladder carcinoma (G3; pT4) with conspicuous pseudoglandular morphology* (**a**, *H&E*), *exhibiting UP III staining in the lumen-lining tumor cell membranes* (**b**). **c**: *Predominantly solid invasive bladder carcinoma (G3; pT3b) with only occasional small lumina* (**arrow** *and* **inset**) *being strongly outlined by UP III antibodies.* **d**: *Another G3 bladder tumor (pT1) exhibiting scattered intracytoplasmic microlumina* (**arrows**). **e** *and* **f**: *G3 bladder tumor (pT2) growing in anastomosing trabeculae* (**e**, *H&E*) *that exhibit membrane staining for UP III along the basal (peripheral) side of tumor cells, apparently resulting in the formation of gaps* (**asterisks**) *to the stroma. Magnifications:* **a** *to* **c**, ×*180;* **inset** *in* **c**, ×*450;* **d**, ×*280;* **e** *and* **f**, ×*300.*

excluded. In some cases, almost complete circumferential peripheral staining and the detachment of small tumor cell nests suggested a pseudopapillary pattern (see below, Figure 7). In 5 cases, *intercellular* membrane staining and/or *cytoplasmic* reactivity for UP III was focally observed. Squamous metaplastic areas of transitional cell carcinomas did not exhibit any UP III staining.

Of the 31 lymph node metastases of transitional cell carcinomas tested (Table 1), 19 were UP III positive (61%; Table 2). The staining patterns for these tumors, which are listed in detail in Table 2, were very similar to those described for invasive primary transitional cell carcinomas (Figure 5). In some of the metastases, the luminal UP III pattern was predominant (Figure 5, a and b). One particular

Table 2. *Uroplakin III in Metastases of Transitional Cell Carcinomas of the Urinary Tract**

Case	Lymph node (N)/ distant (M) metastasis	Grade	UP III pattern†			
			Luminal	Peripheral	Intercellular	Cytoplasmic
1	N	2	+	+	−	−
2–3	N	2	+	−	−	−
4	N	2	−	+	(+)	(+)
5	N	2	−	+	−	−
6–7	N	2	−	−	−	−
8	N	3	+	+	(+)	+
9	N	3	+	(+)	−	(+)
10–11	N	3	(+)	+	−	−
12	N	3	(+)	+	−	−
13–14	N	3	(+)	(+)	−	−
15–16	N	3	(+)	−	−	−
17–18	N	3	−	+	−	−
19–20	N	3	−	(+)	−	−
21–27	N	3	−	−	−	−
28	N	4	−	+	−	−
29–31	N	4	−	−	−	−
32	M (bone)	3	+	−	−	−
33	M (peritoneum)	3	(+)	+	−	−
34	M (omentum majus)	3	−	+	−	−
35	M (liver)	3	−	(+)	(+)	(+)

*As determined immunohistochemically on paraffin sections. Primary tumors were localized in the bladder, in the ureter (case 6), or in the renal pelvis (case 12).
†+, abundant or focal but still moderately numerous positive structures; (+), few or sparse positive structures.

case was characterized by large debris-filled lumina lined by flattened tumor cells (Figure 5c); interestingly, the apical linear UP III staining of these lumen-lining cells resembled that of normal umbrella cells (Figure 5d). Another case of lymph node metastasis had a conspicuous morphology, in that it contained relatively large tumor cell complexes with central areas of clear cells as well as the formation of prominent, partially confluent lumina that proved to be UP III positive (Figure 5, e and f). The invasive portion of the corresponding primary bladder tumor (papillary, G2) exhibited similar staining. Other cases showed predominantly or exclusively the peripheral staining pattern (not shown). In fact, this pattern was even more frequently observed in the metastases as compared with primary tumors (Tables 1 and 2).

Some distant metastases of transitional cell carcinomas of the urinary tract were also included in this study. The staining patterns were again similar as those described for invasive primary tumors and lymph node metastases (Table 2). Figure 6 illustrates an example of a peritoneal metastasis of a bladder carcinoma showing abundant peripheral and focal luminal staining.

In some cases, parallel sections were stained by the affinity-purified rabbit antibodies against UP II. These antibodies yielded similar (albeit somewhat more delicate) staining, with identical structures being positive for UP II and UP III in serial sections (Figure 7, a and b).

A large series of carcinomas derived from other organs (n = 177; Table 1) was included in the UP III series. These comprised 106 primary and 71 metastatic carcinomas, including 14 squamous cell carcinomas, 7 adenocarcinomas and 1 small cell carcinoma of the lung, 1 basal cell carcinoma of the skin, 4 squamous cell carcinomas of the head and neck, 44 invasive ductal and 22 invasive lobular breast carcinomas (Figure 8a), 7 ovarian carcinomas, 17 adenocarcinomas (including signet ring cell carcinomas) of the stomach, 14 colorectal adenocarcinomas, 6 adenocarcinomas of bile ducts, 8 adenocarcinomas of the pancreas, 15 renal cell carcinomas (7 of clear cell type, 4 of chromophilic cell type, and 4 of chromophobe cell type), 2 adenocarcinomas of endometrium, 1 squamous cell carcinoma of the uterine cervix, and 14 prostate carcinomas. Particular care was taken to screen these tumors for the possible presence of very focal immunoreactivity, but none of these tumors were found to be positive for UP III. (For rare cross-reactivities of certain carcinomas with one but not both UP III antibodies, which were considered not to be UP III specific, see Materials and Methods).

Discussion

UPs Ia, Ib, II, and III are specialized membrane proteins of the urothelial plaque constituting the AUM,[20–24]

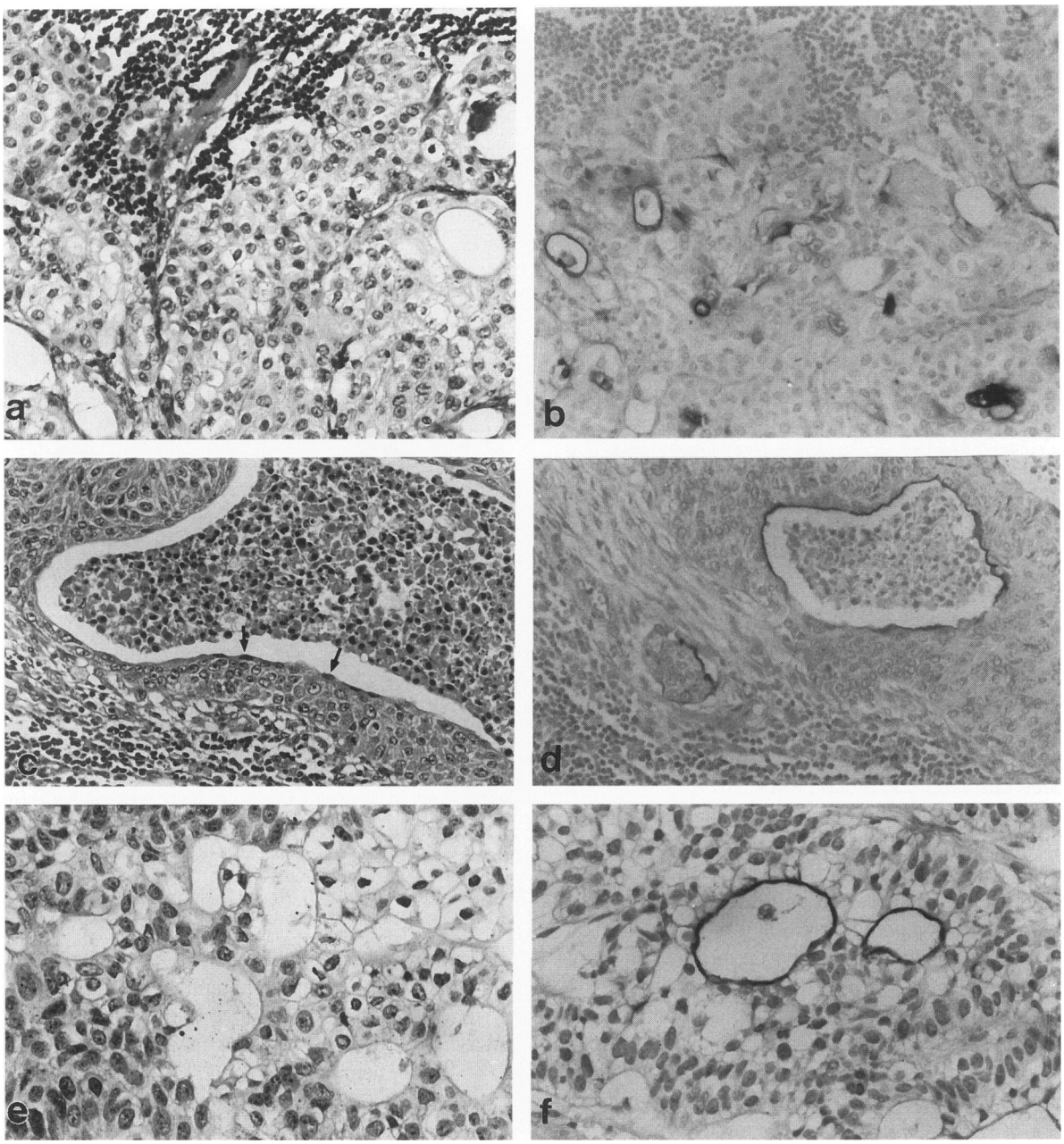

Figure 5. *Detection of UP III in lymph node metastases of transitional cell carcinomas of the urinary bladder.* a, c, *and* e: *H&E staining;* b *and* f: *anti-UP III (A);* d: *anti-UP III (B).* a *and* b: *Typical case (G3; case 9 in Table 2), here showing predominantly luminal UP III staining.* c *to* f: *Cases with special features of UP-III expression.* c *and* d: *Umbrella-cell-like apical UP III staining in flattened tumor cells (some are denoted by* arrows *in* c*) lining large luminal spaces that are filled with debris composed of desquamated necrotic tumor cells (G2; case 3 in Table 2).* e *and* f: *Transitional cell carcinoma variant (G2; case 2 in Table 2) with clear cells* (e, right portion) *and UP III-positive lumina* (f). *Magnifications:* a *to* d, ×*180;* e *and* f, ×*240.*

and they represent the first specific molecular markers of urothelial differentiation (appearing only, in fact, in its terminal stage). With respect to their structural organization and amino acid sequence, UPs are highly conserved among all mammals, including man.[22] The present study demonstrated that UPs II and III are immunohistochemically detectable in routinely prepared paraffin sections of human urothelium, their localization in luminal umbrella cells corresponding to previous findings obtained with frozen sections.[20,21] Moreover, our extensive UP III screening of a variety of normal tissues revealed that the urothelium specificity of this glycoprotein, which, up to now, has only been documented in bovine tissues,[21,25] is indeed valid also

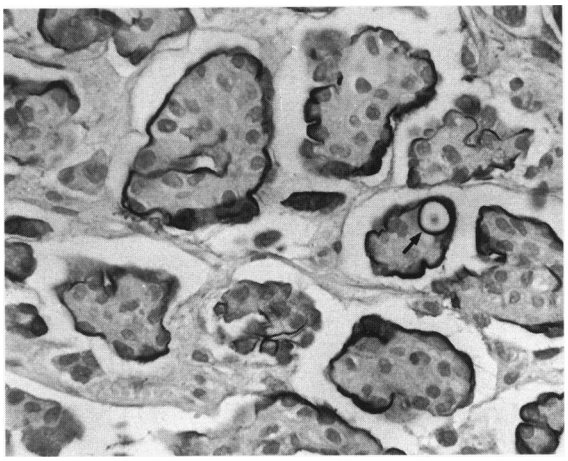

Figure 6. *Strong expression of UP III (anti-UP III (B)), in a peritoneal metastasis of a transitional cell carcinoma of the urinary bladder (G3; case 33 in Table 2). Note abundant cell membrane staining along the basal (peripheral) sides of the tumor cells and formation of gap-like spaces toward the stroma. In addition, UP III staining is seen along a few small lumina* (arrow). *Magnification, ×280.*

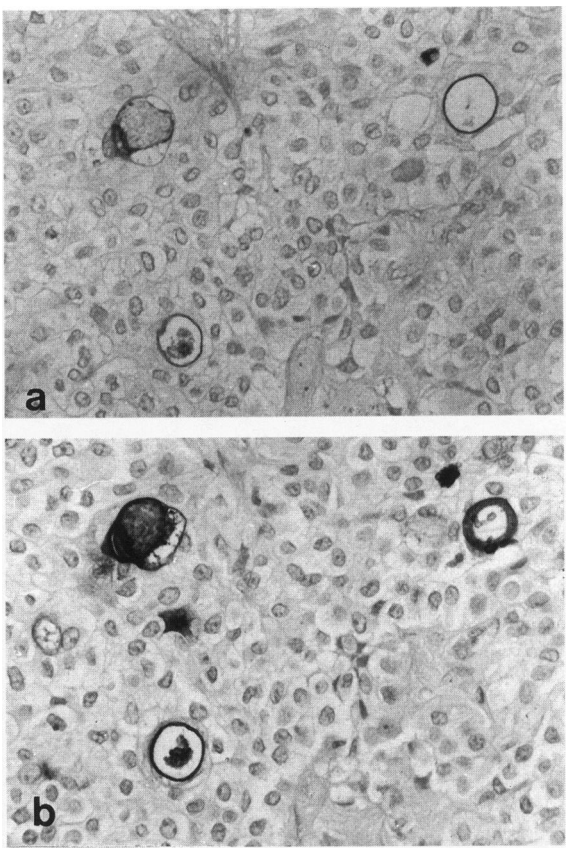

Figure 7. *Comparison of immunostaining for UP II and UP III, exemplarily shown on consecutive sections of a lymph node metastasis of a transitional cell carcinoma of the urinary bladder (G3; case 9 in Table 2).* a: *Anti-UP II.* b: *Anti-UP III (A). Note that the same three lumina exhibit membranous immunostaining for both uroplakins. Magnification, ×280.*

for human tissues. It is of special interest that the specific expression of UP III was detectable in the urothelium of a human fetus at week 13 of gestation; however, the precise time of the first appearance of UPs during human embryogenesis remains to be determined. By electron microscopy, the first appearance of AUM has been observed only around week 21.[29]

Without exception, all nonurothelial tissues studied lacked any detectable UP immunoreactivity. Particularly noteworthy are the negative reactions observed in the epithelial structures located proximally and distally adjacent to the urothelium-lined portion of the urinary tract, ie, the negative staining of the surface epithelium of renal papillae as well as of the pseudostratified epithelium of the membranous urethra, thus indicating that these epithelia lack urothelial differentiation. The same is true of the anal transitional zone epithelium, which morphologically may be reminiscent of urothelium but, on the basis of our results, lacks any relationship with the true transitional epithelium of the urinary tract (see also Ref. 30). Although synthesis of UP Ib appears to occur in a certain lung epithelial cell line (CCL 64, mink cells),[23] we failed to detect UP-positive cells in human lung tissue. It is interesting to note that in the stomach, an organ which, like bladder, may expand tremendously, UPs apparently are not involved in the adjustment of the epithelial surface.

Summerhayes et al[31] have described a series of monoclonal antibodies directed against the urothelium (group III), which produce luminal membrane staining of normal umbrella cells similar to that resulting from UP staining. These group III antibodies are not urothelium specific, however, as they also stain other cells such as pneumocytes. Thus, these antibodies are not related to the UP antibodies tested in the present study but may rather be related to epithelial membrane antigen.[31]

In the screening of a broad range of human carcinomas performed in our study, immunoreactivity for UPs was observed in many transitional cell carcinomas but was not detected in other carcinoma types. Although biochemical confirmation is not yet available, the findings that several rabbit antibodies directed against either UP III or UP II, including affinity-purified preparations, stained exclusively normal urothelial umbrella cells and, in transitional cell carcinomas, produced very similar patterns when applied to serial sections strongly indicate that these antibodies indeed specifically react with UPs in paraffin sections. Occasional cross-reactivities produced by each of the two antisera used against UP III were noted, which concerned mucinous material and cell membranes in certain nonurothelial

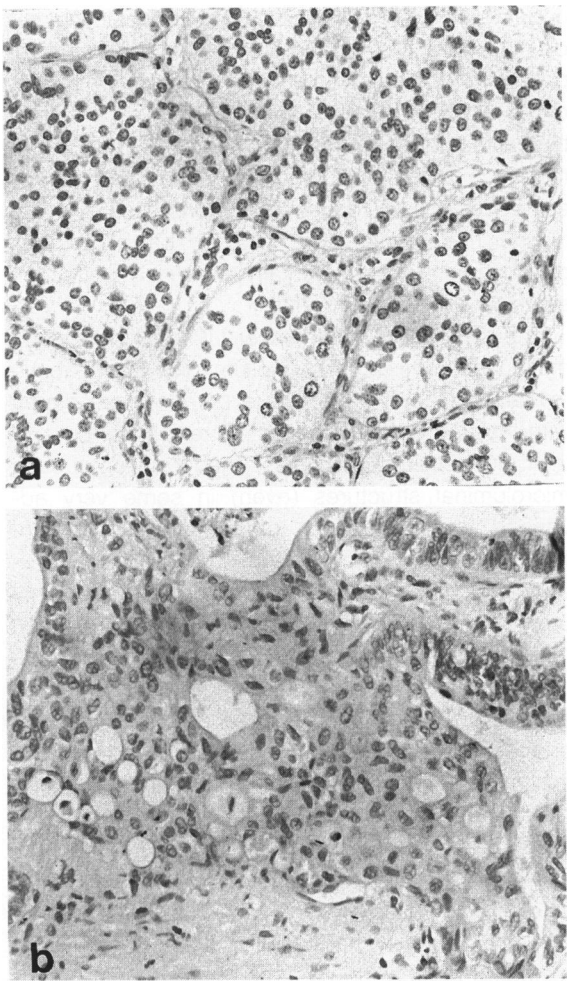

Figure 8. *Examples of UP III-negative carcinoma metastases derived from nonurothelial carcinomas (anti-UP III (A)).* **a**: *Lung metastasis of an invasive ductal carcinoma of the breast.* **b**: *Liver metastasis of an adenocarcinoma of the colon. Magnification, ×180.*

cells and tumors (see Materials and Methods). These cross-reactivities were, however, different for the two antisera and did not coincide in any of the tissues and tumors studied. Therefore, it is likely that the antibodies present in the two antisera recognize different sets of epitopes on the UP III molecule. Staining of the aforementioned nonurothelial tumors may reflect the presence of individual UP III epitopes on molecules either related or unrelated to but certainly distinct from UPs including UP III.

The most important UP III localization patterns in papillary and in invasive transitional cell carcinomas are summarized schematically in Figure 9. In the superficial pattern, UP III is localized, corresponding to the normal urothelium, in the surface membrane of the superficial cells of papillary tumor portions. This pattern may be in correspondence with reports of occasional electron microscopic observations of

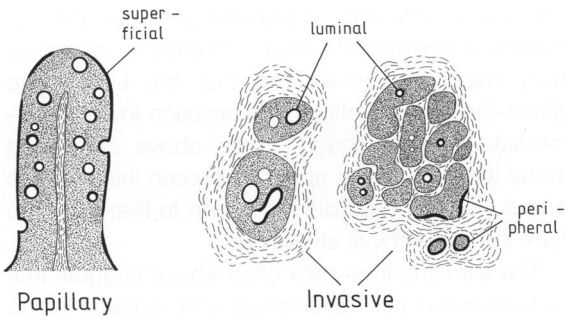

Figure 9. *Schematic drawing of the qualitative UP III staining patterns in transitional cell carcinomas. Thick lines symbolize positive immunohistochemical membrane reaction for UP III.*

AUMs in well differentiated papillary transitional cell carcinomas.[32,33] In contrast, however, Koss[34] and Alroy et al[35] failed to detect any AUM structures in such tumors, and it has further been reported that the surface of such tumors is covered with microvilli[34] rather than the microridges typically associated with normal umbrella cells.[32] Thus, it is likely that UPs are present in the surface membrane of papillary transitional cell carcinomas but are usually not assembled into AUM structures (also see below).

A more unexpected finding was the luminal staining pattern, with UP III being localized in the cell membranes lining variously sized intra- and intercellular lumina, which was found to occur both in papillary carcinomatous epithelium as well as in invasive and metastatic tumor formations (Figure 9). In previous studies, intracytoplasmic lumina present in bladder carcinomas were found, at the ultrastructural level, to be lined by a symmetric unit membrane displaying pleomorphic[36] or small[37] microvilli but not by an AUM. In the lining of intercellular lumina, the presence of a few microvilli and a prominent glycocalyx has been described but again no AUM.[38] At the histochemical level, positive reactions for periodic acid Schiff-diastase and alcian blue (indicating the presence of acid mucins) have been observed along the lining of such lumina as well as in intraluminal amorphous material.[36–38] Interestingly, Delladetsima et al[38] noted that both the intercellular and intracytoplasmic lumina of transitional cell carcinomas exhibited certain features, eg, the presence of secretory component, that were reminiscent of surface umbrella cells, which led them to suggest that the formation of lumina may reflect a focal differentiation of neoplastic urothelial cells toward surface umbrella-like cells. This view is strongly corroborated by our detection of UPs in such lumina. It should be mentioned here that in normal mammalian bladder urothelium AUM plaques have been observed not

only in umbrella cells but also along the apical membranes of intermediate cells.[39] Therefore, the possibility might be considered that the UP-positive gland-like spaces reflect differentiation toward intermediate cells, although in the above mentioned study the intercellular spaces between intermediate and superficial cells did not widen to that extent to form circular luminal structures.

The ultrastructural data cited above suggest that in transitional cell carcinomas UPs present along lumen-forming plasma membranes may not be assembled to form AUMs. Because, as yet, we are unable to correlate the immunohistochemical UP staining pattern of tumors directly with their ultrastructural morphology, the (outside) possibility of the presence of very focally distributed AUMs in some invasive transitional cell carcinomas, which might elude sampling by electron microscopy, cannot be excluded. In fact, in experimental rat bladder tumors, some AUM formation was observed even after invasive carcinoma had developed.[34] But at least one example provides a clear-cut demonstration that UP expression and AUM formation are not necessarily associated, ie, cultured urothelial cells that express all UPs but demonstrably lack AUMs.[40] Even when urothelial cells are cultured at the air-liquid interface to induce differentiation, no AUM is present.[41] Therefore, in human transitional cell carcinomas, it is conceivable that UPs II and III might in fact be integrated into luminal membranes without the development of highly ordered AUM structures. In this context, it should be noted that, throughout various species, intact AUMs always consist of UP Ia, UP Ib, UP II, and UP III,[22] and it is still not clear whether, in normal urothelium or in transitional cell carcinomas, UPs Ia and Ib are co-expressed and co-distributed with UPs II and III.

There is also the possibility that lumina reminiscent of glandular differentiation but exhibiting UP III staining may be a result of inverted lesions producing false luminal or glandular appearing structures. However, such a mechanism clearly would not hold for UP-III-positive lumina in metastatic transitional cell carcinomas.

It is also noteworthy that UP III was often detectable in some but not all of the lumina visible at the light microscope level. Thus, it is conceivable that, in transitional cell carcinomas, the formation of some other kinds of lumina may be related to glandular or secretory processes, this being in accordance with electron microscopic observations of intracytoplasmic lumina being associated with focal glandular differentiation.[36] Theoretically, at least, one cannot completely rule out the possibility that some UP-negative transitional cell carcinomas with lumina that do not exhibit UP staining are in fact false negative, possibly because of the effects of the fixation procedures used. Intracytoplasmic lumina are also very typical features of breast carcinomas,[42] and the fact that such tumors, including those exhibiting lumina, are invariably UP negative demonstrates that the luminal membranes of breast and transitional cell carcinomas are fundamentally different despite their seeming structural similarity.

The localization patterns of UPs in normal and malignant transitional epithelium discussed above reveal that these proteins are primarily associated with the free apical surfaces of transitional cells or their intracellular equivalents. The formation of such microluminal structures (even) in some very advanced and metastatic transitional cell carcinomas,[36–38] for which we have obtained strong evidence that they reflect some degree of true terminal urothelial differentiation, is highly noteworthy. In fact, this finding illustrates that malignant transformation and tumor progression can be compatible with the expression of highly specialized differentiation repertoires, albeit at quantitatively reduced and topologically abnormal levels. Another example of such maintenance of a certain capacity for terminal differentiation is to be found in squamous cell carcinomas, which may focally express the keratinization-related cytokeratins[3] as well as involucrin[14] and filaggrin;[43,44] the latter is characterized by very focal and restricted expression patterns, thereby being comparable to UPs in transitional cell carcinomas. However, filaggrin shows a clear association with the morphological equivalent of squamous differentiation, ie, horny pearls developing centrally in tumor cell nests, whereas in most invasive transitional cell carcinomas morphological evidence of differentiation is hardly discernible even though there may be a considerable level of UP expression.

Given that UP is an apical marker, the finding of a peripheral (basal) localization pattern of UP III in some invasive and metastatic transitional cell carcinomas (Figure 9) was surprising. It might suggest that UPs may, under certain abnormal circumstances, be redistributed in a basal direction. Another way of interpretation could be that these stroma-facing cell membrane domains are actually apical. Whether the gap-like spaces along peripherally stained tumor areas are related in any way to the presence of peripheral UP cannot be decided presently. The possibility remains that these gaps are merely artifacts, eg, from retraction processes during fixation. Additional studies, utilizing electron microscopic techniques are necessary to fully under-

stand this peculiar staining pattern. It also would be important to study the surrounding extracellular matrix proteins. Currently, at least, one can be quite certain that the observed basal pattern genuinely did reflect the specific presence of UPs, as significant staining was seen both with antibodies against UP II and UP III, whereas non-transitional cell carcinomas lacked any basal UP staining.

Up to now, no genuine urothelium-specific marker has been available to assist in the histopathological classification of carcinoma metastases in cases with unknown primary tumors. Although cytokeratin 20 is also a marker of advanced urothelial differentiation, its presence is not as strictly correlated with umbrella cells as that of UPs.[3,45,46] Accordingly, its expression in transitional cell carcinomas is usually more abundant than that of UPs;[3,46] as yet, however, we have not directly compared these two markers on the same tumors. Although the presence of cytokeratin 20 in a carcinoma, particularly when co-expressed with cytokeratin 13, may be indicative of a urothelial origin, the presence of this protein marker can never be taken to be specific for a single organ, as it also occurs in certain other types of epithelia, including gastric and colorectal mucosa, and their tumors.[45,46]

In the present study, UP III has been shown to be the first known specific differentiation and lineage marker of transitional cell carcinomas that can be applied to routinely prepared paraffin sections. It has to be acknowledged that its sensitivity is only moderate, as the expression of UP III may be very focal, but it does seem to be highly specific. Thus, in small biopsy specimens, UP expression might elude observation, so that a negative immunohistochemical result does not necessarily exclude the possibility of a transitional cell carcinoma. However, in the event of positive structures being identified, this can be regarded as a strong indication for a transitional cell origin of a tumor. Similar restricted focal staining, requiring the careful examination of slides, may also occur with other highly specific markers, such as α-fetoprotein for hepatocellular carcinomas or HMB 45 for malignant melanomas.[2] As with these markers, UP III is probably not of value in assessing a tumor's biological behavior or the patient's prognosis, as it is detectable in the majority of transitional cell carcinomas regardless of their grade and stage. Of course, additional studies about the expression of UPs in urothelial carcinomas are required, and these will need to embrace both immunoelectron microscopy and biochemical investigations. Finally, to evaluate possible diagnostic applications of this new marker, the raising of monoclonal antibodies specific for UPs that are suitable for use in paraffin-embedded tissues would be most useful.

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