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Cytokeratins in Normal and Malignant Transitional Epithelium

Maintenance of Expression of Urothelial Differentiation Features in Transitional Cell Carcinomas and Bladder Carcinoma Cell Culture Lines

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The pattern of cytokeratins expressed in normal urothelium has been compared with that of various forms of transitional cell carcinomas (TCCs; 21 cases) and cultured bladder carcinoma cell lines, using immunolocalization and gel electrophoretic techniques. In normal urothelium, all simple-epithelium-type cytokeratins (polypeptides 7, 8, 18, 19) were detected in all cell layers, whereas antibodies to cytokeratins typical for stratified epithelia reacted with certain basal cells only or, in the case of cytokeratin 13, with cells of the basal and intermediate layers. This pattern was essentially maintained in low-grade (G1, G1/2) TCCs but was remarkably modified in G2 TCCs. In G3 TCCs simple-epithelial cytokeratins were predominant whereas the amounts of component 13 were greatly reduced. Squamous metaplasia was accompanied generally by increased or new expression of some stratified-epithelial cytokeratins. The cytokeratin patterns of cell culture lines RT-112 and RT-4 resembled those of G1 and G2 TCCs, whereas cell line T-24 was comparable to G3 carcinomas. The cell line EJ showed a markedly different pattern. The results indicate that, in the cell layers of the urothelium, the synthesis of stratification-related cytokeratins such as component 13 is inversely oriented compared with that in other stratified epithelia where these proteins are suprabasally expressed, that TCCs retain certain intrinsic cytoskeletal features of urothelium, and that different TCCs can be distinguished by their cytokeratin patterns. The potential value of these observations in histopathologic and cytologic diagnoses is discussed. (Am J Pathol 1988, 132:123–144)

AMONG THE EPITHELIAL tissues, the transitional epithelium (urothelium) lining a major part of the urinary tract is characterized by some unique structural and functional features.[1,2] One of the most conspicuous specializations of this multilayered epithelium is the presence of a superficial layer of cells, the "umbrella cells," which contain a peculiar type of vesicles, the "fusiform vesicles," and a thick asymmetrically structured plasma membrane, and a zonula occludens, ie, special structures thought to be involved in the protection of the tissue from urine.[1–4] The notion of some earlier authors[5,6] that not only the basal cells but also the cells of the upper layers are in contact, via thin processes, with the basal lamina has not yet been formally disproved. The urothelium represents a complex system of cells of different types and stages of cell differentiation. Moreover, this tissue has a high

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tendency to change, in response to chemical and/or physical stimuli and perhaps also viral infections, the state of its differentiation to mucous or squamous metaplasia, nonpapillary benign hyperplasias, papillomas, and a broad range of different neoplasias, including squamous cell carcinomas and adenocarcinomas as well as neuroendocrine, spindle, and small cell carcinomas.[2,7-15] The most common type of malignant tumors arising from the urothelium is the group of transitional cell carcinomas (TCCs).[8-13,16] TCCs can be subdivided according to morphologic indications of relative malignant behavior and/or to the criteria of cell differentiation.[8-10,13,16-18] TCCs also present some special problems such as cell-type heterogeneity, high frequency of multifocal lesions and recurrencies, and often show discrepancies between the histologic appearance of a given tumor and its biological behavior. Therefore, urothelial cancer presents a major problem of carcinogenesis and clinical oncology. In addition, cultured carcinoma cell lines of urothelial origin have become important *in vitro* model cell systems for studies of differentiation, transformation, and invasiveness, and are widely used in research on oncogenes.[18-25] Obviously, molecular markers for urothelial differentiation would be of value in studies of normal urothelial development, the various kinds of urothelium-derived tumors, cell sediments from urine,[9] and cultured cells from urine[26-30] or bladder carcinomas.[2,8-17]

The intermediate filament (IF) proteins of the cytokeratin family have been found to be effective markers for the analysis of the type and state of differentiation of epithelial cells.[31-35] The epithelial cytokeratins, comprising a family of at least 19 different polypeptides, are expressed in various combinations, dependent on the route of cell differentiation.[36-41] The synthesis of cytokeratins is usually maintained during malignant transformation, and this feature serves as one of the hallmarks of epithelium-derived tumors,[36-50] including tumors of the urinary tract.[37,41,44-56] One-layered, ie, "simple" epithelia and their carcinomas, usually display a more simple cytokeratin composition than stratified epithelia and squamous cell carcinomas.[36-41,50] The urothelium, as a special, "intermediate-type" epithelium that gives rise to tumors of different degrees of morphologic differentiation, is particularly interesting, and the question of whether different urothelium-derived tumors can be distinguished by their specific cytokeratin patterns is important for the advancement of diagnostic cell typing.

Previous studies of the cytokeratins of normal animal[54,57-60] and human[51,53,56,61] urothelium and bladder carcinomas as well as of cultured urothelial and carcinoma cells[30,62] have been limited by the small spectrum of polypeptide-specific cytokeratin antibodies used and by the number of carcinoma morphotypes examined. Therefore, we have undertaken a systematic study of the cytokeratins present in a large series of urothelial carcinomas, representing the entire range of grades of malignancy and various histologic variants, in comparison with the cytoskeletal composition of diverse cultured bladder carcinoma cells, in a way that relates cytokeratin expression to individual cell types.

Materials and Methods

Various normal human tissues and tumors of the urinary tract, including metastases, were obtained during surgery; in the case of bladder tumors this was done by cystoscopic resection or by cystectomy. Tissue samples were immediately frozen in isopentane precooled in liquid nitrogen to approximately −140 C. Some normal tissues (skin, cornea, spinal cord) obtained at autopsy were used for comparison. The tumors were classified according to WHO standards,[16] using formalin-fixed and paraffin-embedded material and conventional staining procedures. In TCCs 3 grades of malignancy were distinguished.[16]

The permanent bladder carcinoma cell-lines RT-112, RT-4, T-24, and EJ were grown in Weymouth's medium supplemented with 10% fetal calf serum.[19-21,23-25,63] In addition, the following human cell lines were used, the derivation, culture conditions, and IF protein complements of which have been previously described: MCF-7, A-431, PLC, "SV-80 fibroblasts," and U333CG/343MG, a human glioma cell line.[37,41,64,65] Metabolic labelling of cell cultures with ^{35}S-methionine was performed as described.[51]

Immunofluorescence Microscopy

Indirect immunofluorescence microscopy was done on cryostat sections of approximately 5 μ that had been fixed briefly in acetone (precooled to −20 C) and on cultured cells grown on coverslips. The cultured cells had been fixed in methanol (precooled to −20 C) and briefly rinsed in acetone (−20 C). The procedures for single- and double-label immunofluorescence staining have been described.[31-33,51,64,67]

Antibodies

The following murine monoclonal antibodies were used:

a: K_S18.174, K_S18.27, and K_S13.1 are monoclonal antibodies obtained from spleens of Balb/c mice that

had been immunized either with total high-salt-buffer- and detergent-resistant cytoskeletal material of MCF-7 cells (K_S18.174, K_S18.27) or with gel electrophoretically purified cytokeratin 13 from human esophagus (K_S13.1). Fusion and hybridoma selection procedures were essentially according to Köhler and Milstein.[68] Screening of hybridoma supernatants was done by immunofluorescence microscopy on cryostat sections of appropriate human tissues. The characterization and specificities of these antibodies are presented below.

b: CK-2 specific for cytokeratin 18 (commercially available from Boehringer, Mannheim, FRG).[69,70]

c: K_S18.18 reacting with cytokeratin 18 in complexes with basic, ie, type II, cytokeratins.[71]

d: K_S8.1.42 reacting specifically with cytokeratin 8.[72]

e: CK-7 specific for cytokeratin 7 (available from Amersham, U.K.).[73]

f: K_S19.1 (A53-B/A2) reacting exclusively with human cytokeratin 19 (commercially available from Progen Biotechnics, Heidelberg, FRG).[74]

g: KA1 decorating cytokeratin filaments of stratified squamous epithelial and of myoepithelial cells (available from Triton Biosciences, Alameda, CA, USA).[75]

h: KA5 reported to react with cytokeratins 1, 2, 9, 10, and 11.[75]

i: 6B10 specific for cytokeratin 4.[76]

j: 1C7 and 2D7, which are both specific for cytokeratin 13.[76]

k: K_k8.60 specific for cytokeratins 10/11.[77]

l: IVD3A9 was raised and selected as described above for the antibodies mentioned under a using mice immunized with IFs reconstituted from electrophoretically purified human cytokeratins 1 and 14.[78] In immunofluorescence microscopy, this antibody (IgM) reacts with cytokeratin filaments of all stratified squamous epithelia tested. However, while in noncornifying stratified squamous epithelia, such as tongue mucosa, all layers are positive, in epidermis only the basal cells and some suprabasal cells are stained (data not shown). Moreover, the antibody selectively decorates basal cells of prostatic acini and myoepithelial cells of mammary gland ducts. Simple epithelia are negative. Among cultured cells, A-431 cells are heterogenously positive, whereas MCF-7 cells are negative. In immunoblots this antibody reacted with cytokeratin 14 but reactions with other cytokeratins cannot yet be fully excluded.

m: Various antibodies against vimentin (available from Boehringer; clone V9;[79] and Biochrom, Berlin-West; clone VIM-9).

n: Antibodies DE-B-5 specific for desmin[80] and NR4 specific for neurofilament polypeptide NF-L[81] (both available from Boehringer).

o: GF12.24 reacting exclusively with glia filament protein (GFP; available from Progen Biotechnics).[64]

p: DP1 and 2-2.15 against desmoplakins I and II, the major proteins of the desmosomal plaque[82] (available from Boehringer, and Progen Biotechnics).

Guinea pig antibodies against cytokeratins, vimentin or desmoplakins were as described previously.[31-33,42,51,64,82]

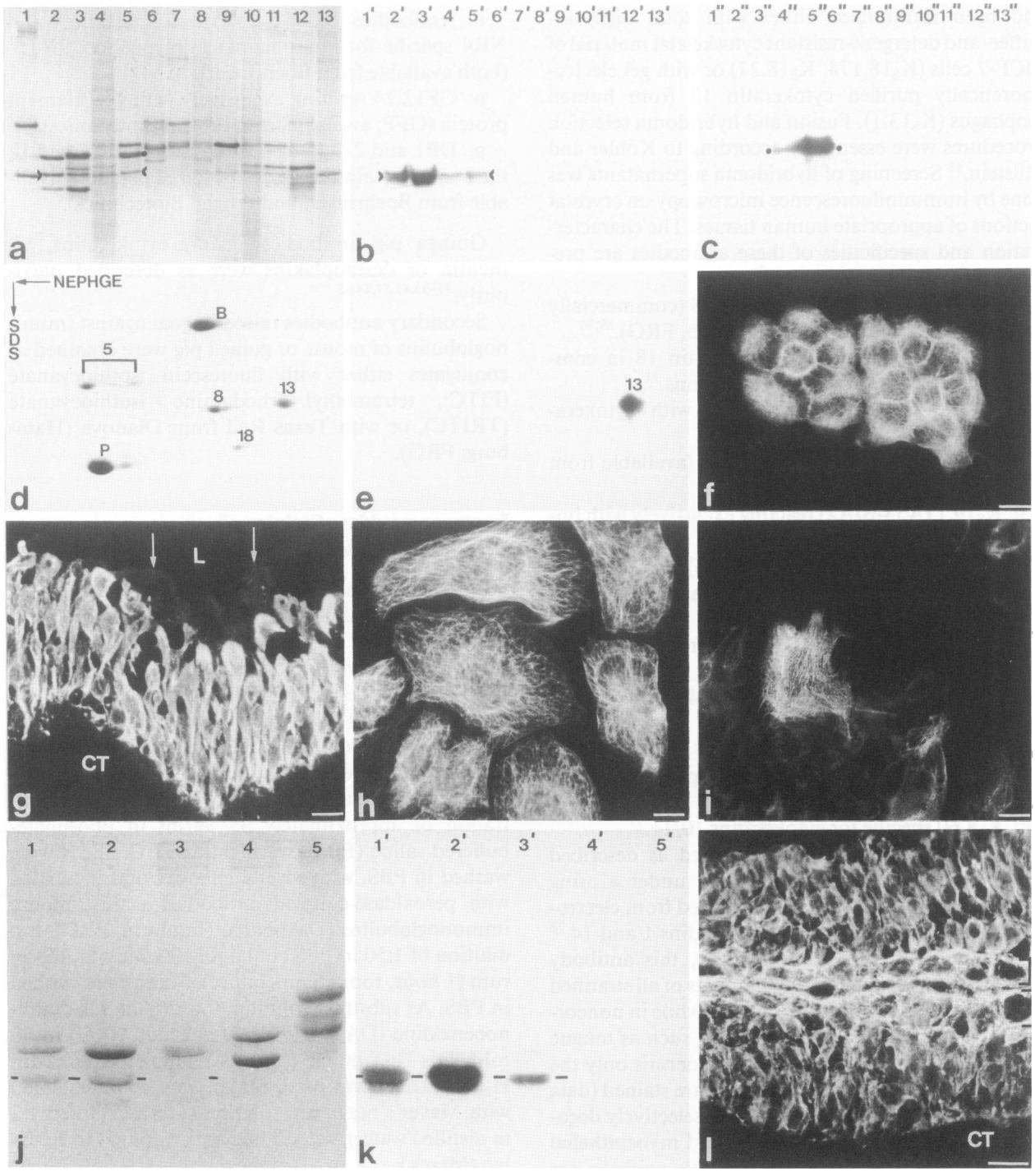
Secondary antibodies raised in goat against immunoglobulins of mouse or guinea pig were obtained as conjugates either with fluorescein isothiocyanate (FITC), tetramethyl - rhodamine - isothiocyanate (TRITC), or with Texas Red from Dianova (Hamburg, FRG).

Immunoperoxidase Staining of Paraffin-Embedded Sections

Suitable antibodies such as K_S13.1 were applied on sections of formalin-fixed tissue, using an indirect immunoperoxidase protocol. 5-μ thick paraffin sections mounted on polylysine-coated slides were dried overnight at 37 C, deparaffinized, and treated with 0.1% protease Type XIV (pronase E from Sigma, St. Louis, MO) in 0.5 M Tris-HCl buffer (pH, 7.4) for 30 minutes at 37 C. After incubation in the same buffer (30 minutes at 4 C), antibodies were applied at a concentration of approximately 35 μg/ml in phosphate-buffered saline (PBS) overnight at 37 C. Slides were washed in PBS, with several changes, and incubated with peroxidase-coupled antibodies against mouse immunoglobulins (Dakopatts, Hamburg, FRG) at a dilution of 1:50 in PBS containing 3% human AB-serum (1 hour, room temperature). Slides were washed in PBS. As substrate solution, 0.6 mg/ml 3,3'-diaminobenzidine (DAB; Merck, Darmstadt, FRG) in 50 mM Tris-HCl buffer (pH, 7.4) supplemented with H_2O_2 (0.01%) was used. Slides were counterstained with Mayer's hemalum solution (Merck; diluted 1:2 in distilled water), dehydrated and mounted in Entellan (Merck).

Preparation of Cytoskeletal Residues From Tissues and Cultured Cells

Epithelial regions of normal and neoplastic tissues were prepared by microdissection of frozen sections under microscopic control as described.[37,61] The preparation of cytoskeletal fractions from both microdis-



sected epithelial tissues and cultured cells was done by extraction in buffers containing Triton X-100 and 1.5 M KCl as described.[65]

Gel Electrophoresis and Immunoblotting

SDS-PAGE and two-dimensional gel electrophoresis of cytoskeletal proteins, autoradiography of gels containing ^{35}S-labeled proteins and immunoblotting procedures were as described.[65] The proteins were stained using either Coomassie Brilliant Blue R 250 or with a silver staining technique. Proteins transferred to nitrocellulose sheets were stained with Ponceau-S (Sigma). Specific antigen–antibody complexes were visualized on autoradiographic film (Ko-

Figure 1—Characterization of monoclonal antibodies K_S18.174 (**a, b**), K_S13.1 (**a, c–i**), and K_S18.27 (**j–l**) by immunoblotting of cytoskeletal proteins and immunofluorescence microscopy. **a**—Reference gel electrophoresis (SDS-PAGE) for the characterization of antibodies K_S18.174 and K_S13.1. Lanes 2–13 show the IF protein complements present in total cell lysates and cytoskeletal fractions from several human cell cultures and tissues (for specific cytokeratin compositions see [37,41]). Lane 1: Reference proteins (from top to bottom): myosin heavy chain (M_r ~ 220,000), bovine serum albumin (BSA, M_r ~ 68,000), α-actin from rabbit muscle (M_r ~ 43,000). Lane 2: MCF-7 cytoskeleton. Lane 3: RT-4 cytoskeleton. Lane 4: A-431, whole cell lysate. Lane 5: A-431 cytoskeleton. Lane 6: Epidermis cytoskeleton. Lane 7: Cornea cytoskeleton. Lane 8: Foot sole epidermis, cytoskeleton. Lane 9: SV40 transformed fibroblasts ("SV80 cells") containing only vimentin IFs; cytoskeleton. Lane 10: Glioma cells, whole cell lysate. Lane 11: Glioma cells, cytoskeleton containing vimentin (M_r ~ 55,000) and GFP (M_r ~ 50,000). Lane 12: Human spinal cord, cytoskeleton containing neurofilament polypeptides NF-H, NF-M, NF-L as well as vimentin and GFP. Lane 13: Human myometrium, cytoskeleton showing vimentin and desmin (M_r ~ 53,000). The dots denote the position of cytokeratin 13, the arrowheads that of cytokeratin 18. **b**—Autoradiogram of a corresponding immunoblot, using antibody K_S18.174 and ^{125}I-labeled secondary goat antibodies to mouse immunoglobulins. Positive reaction of cytokeratin 18 is seen in lanes 2′–5′. **c**—Autoradiogram of a corresponding immunoblot, using antibody K_S13.1. Note specific reaction with cytokeratin 13 in lanes 4″ and 5″. At the exposure time shown, no reaction is seen in lane 3 (RT-4 cells), corresponding to the very low amount of cytokeratin 13 in this cell line. Secondary antibodies were the same as in (**b**). **d**—Two-dimensional gel electrophoresis of A-431 cytoskeletal proteins, using nonequilibrium pH gradient gel electrophoresis (NEPHGE) in the first dimension and SDS-PAGE in the second dimension electrophoresis. Reference proteins for co-electrophoresis are BSA (B) and 3-phosphoglycerokinase from yeast (P, M_r ~ 43,000). **e**—Autoradiogram of a corresponding immunoblot using antibody K_S13.1, showing the specific reaction with cytokeratin 13. **f**—Immunofluorescence micrograph of A-431 cell culture, after reaction with antibody K_S13.1, showing a homogeneous fibrillar staining of all cells. Bar, 20 μ. **g**—Immunofluorescence microscopy on frozen section through human urothelium with antibody K_S13.1, showing bright staining of the basal and intermediate cell layers but absence of reaction in the umbrella cells (apical membrane demarcated by arrows; L, lumen; CT, connective tissue). **h**—Immunofluorescence microscopy of RT-112 cell culture with antibody K_S13.1, showing bright fibrillar staining. **i**—Immunofluorescence microscopy of RT-4 cell culture with antibody K_S13.1 showing that only a few of the cells are positive. **j**—Reference SDS-PAGE of cytoskeletal proteins from various human cell cultures and tissues, used for the characterization of monoclonal antibody K_S18.27. Experimental conditions were as in **a**. Lane 1: PLC cytoskeletons (from top to bottom: vimentin, cytokeratins 8, and 18). Lane 2: MCF-7. Lane 3: RT-112. Lane 4: Human glioma cell line. Lane 5: Human epidermis. **k**—Autoradiogram of the corresponding immunoblot with antibody K_S18.27, showing the specific reaction with cytokeratin 18 (experimental conditions as in **b, c,** and **e**). **l**—Immunofluorescence microscopy of cryostat section of urothelium of human ureter with antibody K_S18.27, showing strong staining of the umbrella cells and moderate staining of the lower urothelial cell layers. Scale bars, 20 μ.

dak X OMAT AR5, Kodak, Stuttgart, FRG) using ^{125}I-labeled, species-specific secondary antibodies or ^{125}I-labeled protein A.

Results

Characterization of Antibodies K_S18.174, K_S18.27, and K_S13.1

These three monoclonal antibodies, which were of particular value in the present study, belong to the IgG1 subclass. When examined by immunoblotting on the complete spectrum of IF polypeptides 2 of them, K_S18.174 (Figure 1a, b) and K_S18.27 (Figure 1j, k), reacted exclusively with cytokeratin 18, whereas antibody K_S13.1 showed a strong reaction with cytokeratin 13 (Figure 1a, c–e) and, after extremely prolonged exposure, also weak reactions with cytokeratins 14 and 16 (not shown).

By immunofluorescence microscopy, typical cytokeratin fibril staining was obtained with antibody K_S13.1 in all cells of A-431 cultures (Figure 1f) and in basal and intermediate cell layers but not in umbrella cells of urothelial tissue (Figure 1g) as well as in most but not all cells of RT-112 cultures (Figure 1h). In contrast, only few individual cells of the RT-4 cell line were positive (Figure 1i). Antibody K_S13.1 was reactive with formaldehyde-fixed, paraffin-embedded tissue, producing the same staining patterns of normal urothelium and transitional cell carcinomas as frozen tissue (for example, see Figure 2a, b).

When examined on cryostat sections of diverse human tissues, monoclonal antibodies K_S18.174 and K_S18.27 immunostained all simple-type and complex epithelia, including urothelium of the bladder (Figure 3b for K_S18.174; Fig. 1l for K_S18.27) but did not react on epidermis and other stratified squamous epithelia, with the exception of certain basal-cell-layer and suprabasal regions weak staining in some samples from the esophagus and vagina that were also shown to be positive for simple epithelium-type cytokeratins 8 and 18 by *in situ* hybridization. Neither of the 2 antibodies reacted with nonepithelial cells of the connective tissue, skeletal and cardiac muscle, and nervous tissue. On cultured cells, filamentous staining was seen only on epithelial cells (for details, see below). Both monoclonal antibodies reacted with different epitopes as has been determined by epitope mapping. The epitope of K_S18.27 is located in the chymotrypsin-resistant α-helical, central rod domain whereas that recognized by

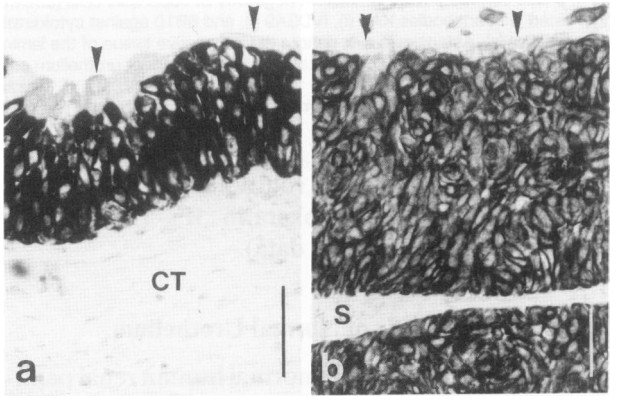


Figure 2—Immunoperoxidase staining (formalin-fixed, paraffin-embedded material) of normal renal pelvis urothelium (**a**) and papillary transitional cell carcinoma (G1) of renal pelvis (**b**) using antibody K_S13.1. Note that the staining patterns are similar to those obtained with frozen sections (see Figures 1g, 3, and 4). Arrowheads in **a** and **b** denote unstained superficial (in **a**: "umbrella") cells; CT, connective tissue of *lamina propria;* S, stroma of tumor papilla. Bars, 50 μ.

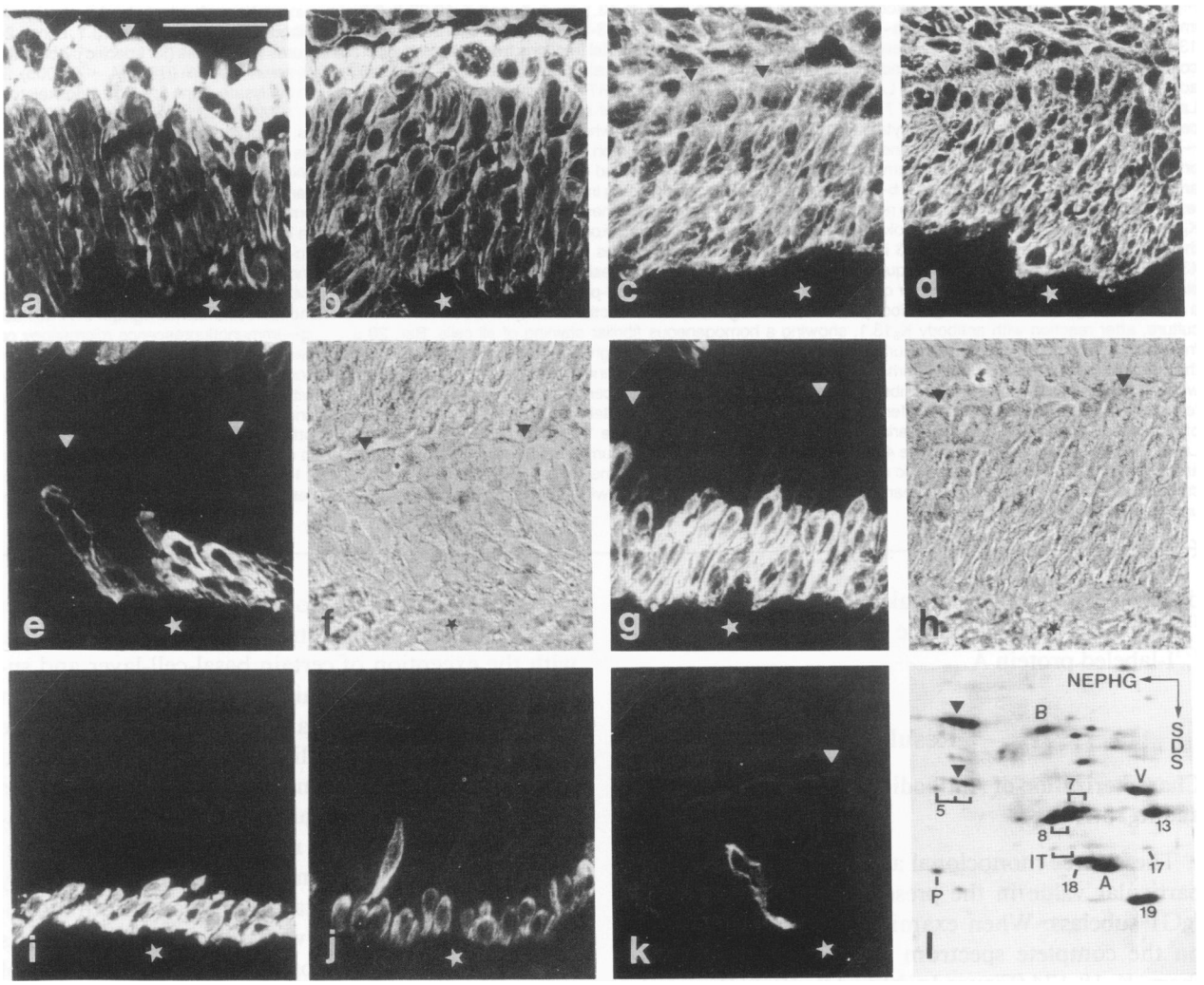


Figure 3—Immunofluorescence microscopy and gel electrophoresis of cytokeratins of normal human transitional epithelium. **a–d**—Urothelium of renal pelvis (**a, b**) and ureter (**c, d**) stained with antibodies K_S8.1.42 against cytokeratin 8 (**a**), K_S18.174 against cytokeratin 18 (**b**), CK-7 against cytokeratin 7 (**c**), and K_S19.1 against cytokeratin 19 (**d**). Note that all antibodies stain all epithelial cell layers but that the antibodies against cytokeratins 8 and 18 decorate the superficial (umbrella) cells (arrowheads) with particularly high intensity (**a, b**). **e–h**—Antibody 2D7 against cytokeratin 13, showing a limited staining in renal papilla surface epithelium (**e; f**, corresponding phase contrast micrograph) but a more extended staining in the better developed renal pelvis urothelium (**g; h**, phase contrast). Note consistent negativity of superficial cells (arrowheads). **i–k**—Renal pelvis urothelium (**i, j**) and renal papilla surface epithelium (**k**) stained with antibodies KA1 (**i**), IVD3A9 (**j**), and 6B10 against cytokeratin 4 (**k**), all staining certain cell populations in the basal cell layer (arrowhead in **k**, superficial cells). Asterisks in **a–k** denote the connective tissue of the lamina propria which is negative with all antibodies used here. Bar (in **a**), 50 μ. **l**—Cytoskeletal polypeptides of microdissected human bladder urothelium as revealed by two-dimensional gel electrophoresis (silver staining; for methods see text). Cytokeratins are designated by their catalog numbers.[37] IT, IT protein; V, vimentin from cells of the lamina propria; A, actin; arrowheads, unidentified cytoskeletal polypeptides, perhaps lamins A (upper) and C (lower arrowhead). For further designations see legend to Figure 1d.

K_S18.174 is lost from the rod domain after chymotrypsin digestion of cytokeratin IFs (Bruder, G, Franke, WW, unpublished data).

Cytokeratin Patterns of Normal Urothelium

When frozen sections of normal human renal papillae, renal pelvis, ureter, and urinary bladder, including the urothelial lining, were reacted with the various cytokeratin antibodies different patterns of epithelial immunostaining were observed with different cytokeratin antibodies (Figure 3a–k). In urothelium of all sites, antibodies against each of the 4 simple-epithelium-type cytokeratins 7 (CK-7), 8 (K_S8.42), 18 (K_S18.174, K_S18.27, and K_S18.18), and 19 (K_S19.1) reacted with the entire transitional epithelium (Figure 3a–d). While the superficial ("umbrella") cells, which were present in all regions with the exception of the renal papillae, were strongly positive for all these antibodies, the basal and intermediate cells were stained intensely only by CK-7 and K_S19.1 (Figure 3c, d) but were only moderately reactive with the other antibodies (Figure 3a, b).

With antibodies 1C7 and 2D7 against cytokeratin

13, a radically different, nonuniform staining pattern of the urothelium was seen (Figure 3e–h). In most regions of the primitive urothelium lining renal papillae, cytokeratin 13 staining was confined essentially to scattered or clustered basal cells and was often of only weak-to-moderate intensity (Figure 3e, f). The fully developed urothelium of renal pelvis, ureter, and urinary bladder showed a more extended staining of the basal and intermediate cell layers. The whole basal-cell layer and the lower part of the intermediate layer (Figure 3g, h), often even most of the intermediate layer cells (Figure 1g), were cytokeratin 13-positive. There was, however, some heterogeneity between different tissue samples and different areas in a given sample. Occasionally, regions were encountered in which cytokeratin 13 was detected only in clusters of basal cells similar to the situation in renal papillae epithelium. Usually, however, only the umbrella cells were negative for cytokeratin 13 (Figure 3g, h). Essentially the same pattern was produced with antibody K_S13.1 (Figures 1g and 2a).

Antibody KA1, directed against cytokeratins of stratified squamous epithelia, specifically stained the basal cells of the urothelium at all sites studied (Figure 3i), and a similar, though less intense and uniform, reaction was seen with antibody IVD3A9 (Figure 3j). Cytokeratin 4 antibody 6B10 stained rare individual cells in basal positions or in intermediate positions but with contact to the basal lamina (Figure 3k). Cytokeratin 10/11 antibody K_k8.60 yielded negative reactions (not shown).

The urothelium was negative with vimentin antibodies that, in turn, stained the nonepithelial cells of the underlying tissue (not shown). Similarly, the urothelium was negative for desmin, GFP, and NF-L (not shown).

Two-dimensional gel electrophoresis of microdissected urinary bladder tissue enriched in urothelium showed relatively large amounts of the simple-epithelium-type cytokeratins 7, 8, 18, and 19 and of the stratified-epithelium-type cytokeratin 13 (Figure 3l). In addition, minor amounts of cytokeratins 5 and 17, and of the as-yet uncharacterized cytoskeletal protein "IT" described previously in intestinal epithelium, colon carcinoma, and Merkel cell tumor cells[37,83] could be identified (Fig. 3l).

Urothelial Carcinomas

Twenty-two primary or metastatic human carcinomas, mostly TCCs, of the urothelium of the urinary bladder, the ureter, and the renal pelvis were analyzed by immunofluorescence microscopy and gel electrophoresis. Clinical and pathologic data and a summary of the results are presented in Table 1, which also allows a comparison with normal urothelium and several bladder carcinoma cell lines (see below).

In all cases, cytokeratin proteins were detected in virtually all tumor cells (Figures 4–7) which, in turn, were negative for vimentin (Figure 4k), with the exception of 1 case that contained some vimentin-positive tumor cells (Table 1). All tumors were strongly positive for desmoplakin, showing the typical punctate arrays of desmosomal staining (data not shown; cf. ref.[66]), in agreement with the relatively high frequency of desmosomes in human and animal bladder carcinomas determined by electron microscopy.[2] Some cases were tested immunocytochemically for desmin, GFP, and neurofilament protein NF-L; the tumor cells were always negative whereas smooth muscle cells were positive for desmin and small nerves were positive for the two other IF protein types (not shown). A cytoskeletal protein designated IT[37,83] was found biochemically in small amounts in most (18 out of 22) but not all urothelial tumors (Figure 4l, Figure 5d, h, n; Figure 6f).

Common features as well as marked differences of expression and cellular distribution of the various cytokeratin polypeptides were noted between different cases. These cytokeratin phenotypes could be arranged in certain basic patterns.

In all pure TCCs, gel electrophoresis showed all 4 simple-epithelium-type cytokeratins 7, 8, 19, and 19, together with variable amounts of certain stratified-epithelial cytokeratins, the proportion of which was always lower than that of the simple-epithelial cytokeratins (Table 1).

In low-grade (G1 and G1/2) papillary TCCs (Figure 4a, e) antibody K_S18.174 against cytokeratin 18 decorated all tumor cells (Figure 4b, f). Superficial cells resembling umbrella cells, which lined the surface of papillae or slitlike lumina of deeper tumor cell nests, sometimes showed a higher staining intensity (Figure 4b). Uniformly positive staining was also seen with the antibody against cytokeratin 19 (not shown). All 3 antibodies against cytokeratin 13 (2D7, 1C7, K_S13.1) decorated the tumor cells uniformly (Figure 4c, g), with the exception of the superficial umbrella-celllike cells that exhibited variable immunoreactivity (Figure 4c). In contrast, antibody KA1 revealed a markedly heterogenous staining, with the positive cells being enriched in basal layers (Figure 4h). Antibody IVD3A9 was positive in some regions, displaying a pattern similar to that of antibody KA1 (Figure 4i). Antibody 6B10 against cytokeratin 4 usually stained a few isolated tumor cells (Table 1). The antibody against cytokeratins 10/11 stained only occasional cells in only one of the cases (Table 1), whereas all other TCCs

Table 1—Expression of Stratification Related Cytokeratins in Normal and Malignant Urothelial Cells, Including Clinical and Pathologic Data of the Tumors Studied

Case	Age, sex	Site*	Histology	Grade	Stage/ invasion type†	Immunofluorescence microscopy‡						Gel electrophoresis§												Cytokeratin pattern type
												Stratified-epithelium-type cytokeratins								Simple-epithelium-type cytokeratins				
						K_K8.60 (10/11)	IVD3A9	6B10 (4)	1C7/2D7 (13)	K_S18.174 (18)	Vimentin	4	5	6	10‖	13	14	16	17	7	8	18	19	
Pure transitional cell carcinomas (TCCs)																								
1	78 f	B	P TCC	1	Ta	–	–	(+)¶	++**	++††	–	–	(+)	–	–	+	–	–	–	+	+	(+)	+	A
2	54 m	B	P TCC	1	Ta	–	(+)¶	(+)	++	++	–	–	tr	–	–	+	–	–	(+)	+	+	(+)	+	A
3	64 f	U	P TCC	1	Ta	(+)¶	(+)B	+B	++	++	–	(+)	–	–	–	+	–	–	(+)	+	+	(+)	+	A
4	71 m	B	P TCC	1/2	T1	–	+B	(+)	++‡‡	++§§	–	–	+	tr	–	+	tr	–	(+)	+	+	(+)	+	A
5	65 m	U	P TCC	1/2	T2‖‖	–	(+)B	–	++**	++††	–	–	(+)	–	–	+	(+)	–	(+)	+	+	(+)	+	A
6	44 m	B	P TCC	2	T1	–	(+)B	(+)B¶	+B	++	–	–	+	–	–	tr	–	–	(+)	+	+	(+)	+	B
7	43 m	B	P TCC	2	T1	–	(+)B***	(+)	(+)	++	–	–	–	–	–	tr	–	–	+	+	+	(+)	+	C
8	56 m	B	P TCC	2†††	T1	–	+B	(+)	+	++	–	–	tr	–	–	(+)	–	–	(+)	+	+	(+)	+	D
9	57 m	B	P TCC	2‡‡‡	T3b	–	(+)B	+	+	++	–	–	tr	–	–	(+)	–	–	(+)	(+)	+	tr	+	D
10	62 m	Re	P TCC	2/3	T1	(+)¶	(+)(B)§§§	(+)	+	++	–	–	(+)	tr	–	tr	–	–	(+)	+	+	(+)	+	D
11	56 m	B	P TCC	3	T1	–	(+)¶	(+)(B)§§§	+(B)§§§	++	–	–	–	–	–	tr	–	–	(+)	+	+	(+)	+	D
12	64 m	B	Non-P TCC	3	T3b	–	–	(+)¶	(+)	++	–	–	–	–	–	tr	–	–	tr	+	+	(+)	+	E
13	66 m	B	P TCC	3	N	ND	ND	ND	(+)	++	ND	–	–	–	–	–	–	–	tr	+	+	+	(+)	E
14	64 m	B	P TCC	3‖‖‖	N	–	–	(+)¶	(+)(B)§§§	++	–	–	–	–	–	–	–	–	(+)	+	+	+	+	E
15	67 m	B	Non-P TCC	3	T2¶¶¶	–	–	(+)¶	(+)¶	++	–	–	–	–	–	–	–	–	–	+	+	(+)	+	E
16	68 m	B	P TCC with glandular metaplasia	3	T1	–	–	–	(+)B****	++	–	–	–	–	–	–	–	–	tr	+	+	(+)	+	E
17	29 f	B	Non-P TCC	3	N	–	–	(+)	–	++‡‡	–	–	–	–	–	–	–	–	+	(+)	+	+	(+)	E
18	59 f	B	Non-P TCC	3	M (skin)	–	ND	ND	–	++	–	–	–	–	–	–	–	–	(+)	+	+	+	+	E

Carcinomas with squamous features																							
19	82 f	B	TCC with low squamous tendency††††	3	N	–	(+)	++	++	++	–	+	tr	+	–	+	tr	(+)	+	+	+	(+)	+	F
20	84 m	B	P TCC with SM	2/3	T1	(+)	++	++‡‡	++‡‡‡	++	–	tr	+	+	–	+	tr	tr	(+)	(+)	+	(+)	+	F
21	61 m	Re	P TCC with SM	2/3‡‡‡‡	T4	+	+B	+	++	++††‡‡	–	–	+	(+)	(+)	+	–	–	(+)	+	(+)	(+)	(+)	F
						(+)	+	–	+	++‡‡	+													
22	60 m	B	Poorly differentiated SCC	3	T3a	–	++‡‡	(+)	+	++‡‡	–	–	+	+	–	(+)	+	–	+	(+)	(+)	(+)	+	G
Normal urothelium						–	(+)B	(+)B¶	+B	++§§§§	–	–‖‖‖‖	(+)	–	–	+	–	–	tr	+	+	+	+	
Cell line																								
RT-112						–	–	(+)	++‡‡	++	–	tr	(+)	tr	–	+	–	–	+	+	+	+	+	
RT-4						–	–	(+)¶	(+)	++	–	–	–	–	–	(+)	–	–	+	+	+	+	+	
T-24						–	–	–	–	++	++	–	–	–	–	–	–	–	(+)	+	+	+	(+)	
EJ						–	–	–	–	+	++	–	–	–	–	–	–	–	–	–	+	+	–	

B, bladder; U, ureter; Re, renal pelvis; P, papillary; TCC, transitional cell carcinoma; SCC, squamous cell carcinoma; SM, squamous metaplasia.

* When metastatic lesions were examined, the site of the primary tumor is indicated.

† Stage refers to the lesion examined. T, primary tumor examined. Bladder, ureter and renal pelvis tumors are staged according to the UICC scheme: Ta, papillary and noninvasive; T1, invasion of the subepithelial connective tissue; T2, invasion of the superficial muscle layer (bladder tumors) or the muscle layer (ureter and renal pelvis tumors); T3a, invasion of the deep muscle layer; T3b, invasion of the perivesical fatty tissue; T4, invasion of the perirenal fatty tissue (renal pelvis tumor). N, regional lymph node metastasis examined. M, distant metastasis examined.

‡ Cytokeratin specificities are indicated in brackets (for antibody IVD3 A9 see text). ++, uniformly positive reaction of most or all cells. +, heterogenously positive reaction. (+), few cells positive. B, basal (peripheral) pattern with positive cells mainly located in the basal cell layer or at the periphery of tumor cell nests. ND, not done.

§ +, prominent relative amount. (+), minor relative amount. tr, trace amount. –, protein not detected.

‖ In case 21, the authors could not distinguish between cytokeratins 10 and 11.

¶ Positive cells are very rare.

** Some of the covering cells present in some areas are negative.

†† Covering cell layer present in some areas more strongly stained.

‡‡ Few cells negative.

§§ In some areas, peripheral cells are more weakly stained.

‖‖ Invasion of muscle layer occurred mostly *en bloc*.

¶¶ Positive tumor cells only present in some areas.

*** In addition to heterogenously and weakly positive basal (peripheral) cells, scarce and scattered nonbasal cells were strongly positive.

††† A small part of this case was G3 (not studied here) and superficially invaded the prostate.

‡‡‡ A part of this case was G3 (not studied here).

§§§ Among the positive tumor cells, some were in peripheral and some in nonperipheral location.

‖‖‖‖ Primary tumor grade 2.

¶¶¶ The tumor infiltrated the muscle layer but the depth of infiltration could not be determined.

**** Only in one small area, flat peripheral tumor cells were positive.

†††† The lymph node metastasis studied morphologically exhibited focal, poorly developed squamous features.

‡‡‡‡ This tumor, which infiltrated the renal parenchyme and the perirenal fatty tissue, exhibited some G2 areas (upper symbols in immunofluorescence columns), focally intermingled with high-grade parts (G3, tentacular invasion; lower symbols in immunofluorescence columns).

§§§§ Umbrella cells more strongly stained.

‖‖‖‖ In some samples, traces of cytokeratin 4 were found.

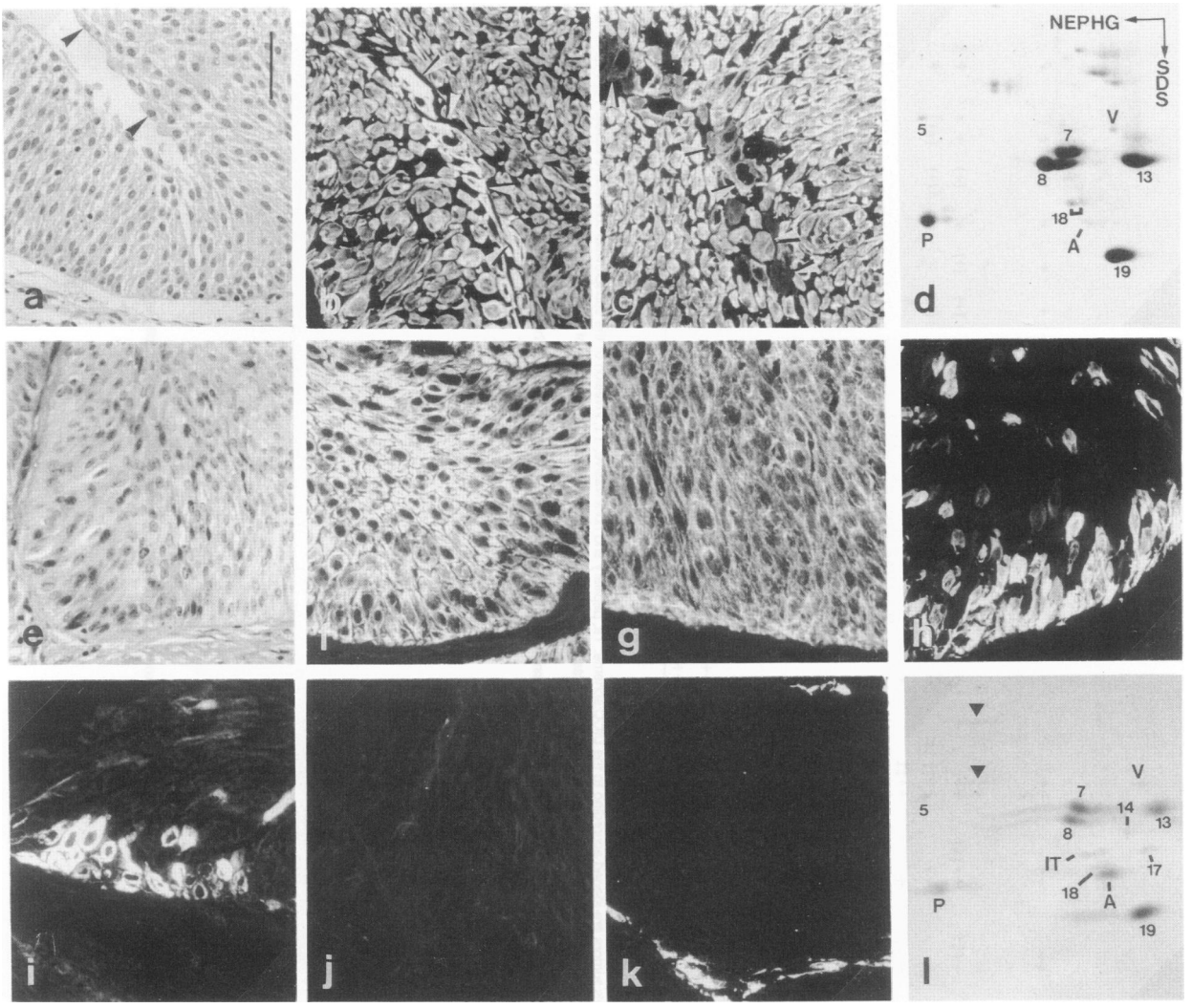


Figure 4—Cytokeratins of low grade (G1, G1/2) transitional cell carcinomas, demonstrated by immunofluorescence microscopy and gel electrophoresis. **a–d**—Papillary transitional cell carcinoma (TCC) of the bladder, G1, non-invasive (case no. 1). **a**—H & E staining (paraffin-embedded material). **b, c**—Immunofluorescence microscopy (frozen sections) with antibodies K_S18.174 against cytokeratin 18 (**b**) and 2D7 against cytokeratin 13 (**c**). Note that the superficial cells still discernible in this tumor (arrowheads in **a–c**) are particularly strongly stained for cytokeratin 18 (**b**) but that some of them are negative for cytokeratin 13 (**c**). **d**—Gel electrophoretic cytokeratin pattern (Coomassie Blue staining). Note prominent amount of cytokeratin 13. **e–l**—Papillary TCC of ureter, G1/2 (case no. 5). **e**—H & E staining (as in **a**). **f–k**—Immunofluorescence microscopy showing that the deep solid tumor cell pegs are uniformly positive for cytokeratin 18 (**f**; antibody K_S18.174) and cytokeratin 13 (**g**; antibody 1C7) but heterogenously positive with basal enrichment with antibodies KA1 (**h**) and IVD3A9 (**i**) and negative with antibody KA5 (**j**) and the vimentin antibody VIM-9 (**k**); note that the latter stains stromal cells. **l**—Gel electrophoresis (Coomassie Blue staining) revealing a pattern similar to that of the G1 case shown in **d**. For designations in **d** and **l** see also legend to Figure 3l. Bar (in **a**), 50 μ.

were negative (Figure 4j). The tumor cells were negative for vimentin (Figure 4k).

Gel electrophoresis of microdissected low-grade TCC tissues disclosed major amounts of cytokeratins 7, 8, 19, and 13 as well as, in most or all cases, minor amounts of cytokeratins 5, 17, and 18 (Table 1; Figure 4d, l). Small amounts of IT protein were also noticed in most analyses (Figure 4l). The basic cytokeratin pattern common to all low-grade carcinomas studied, which is characterized by the prominence of cytokeratins 7, 8, 19, and 13 with some cytokeratin 18 and a nearly uniform distribution of cytokeratin 13, was designated "pattern A." There were, however, some minor variations, primarily in the relative amount of cytokeratin 5 (cytokeratin 4 has not been detected in most of our biochemical analyses, probably due to its low concentration) and the extent of the staining with antibodies IVD3A9 and 6B10 (Table 1).

Among papillary TCCs of grade G2, 3 basic patterns could be distinguished, all of which showed reduced amounts of cytokeratin 13 compared with the low-grade tumors and a heterogenous distribution of cells positively stained by cytokeratin 13 antibodies. One papillary G2-tumor (Figure 5a), which was uni-

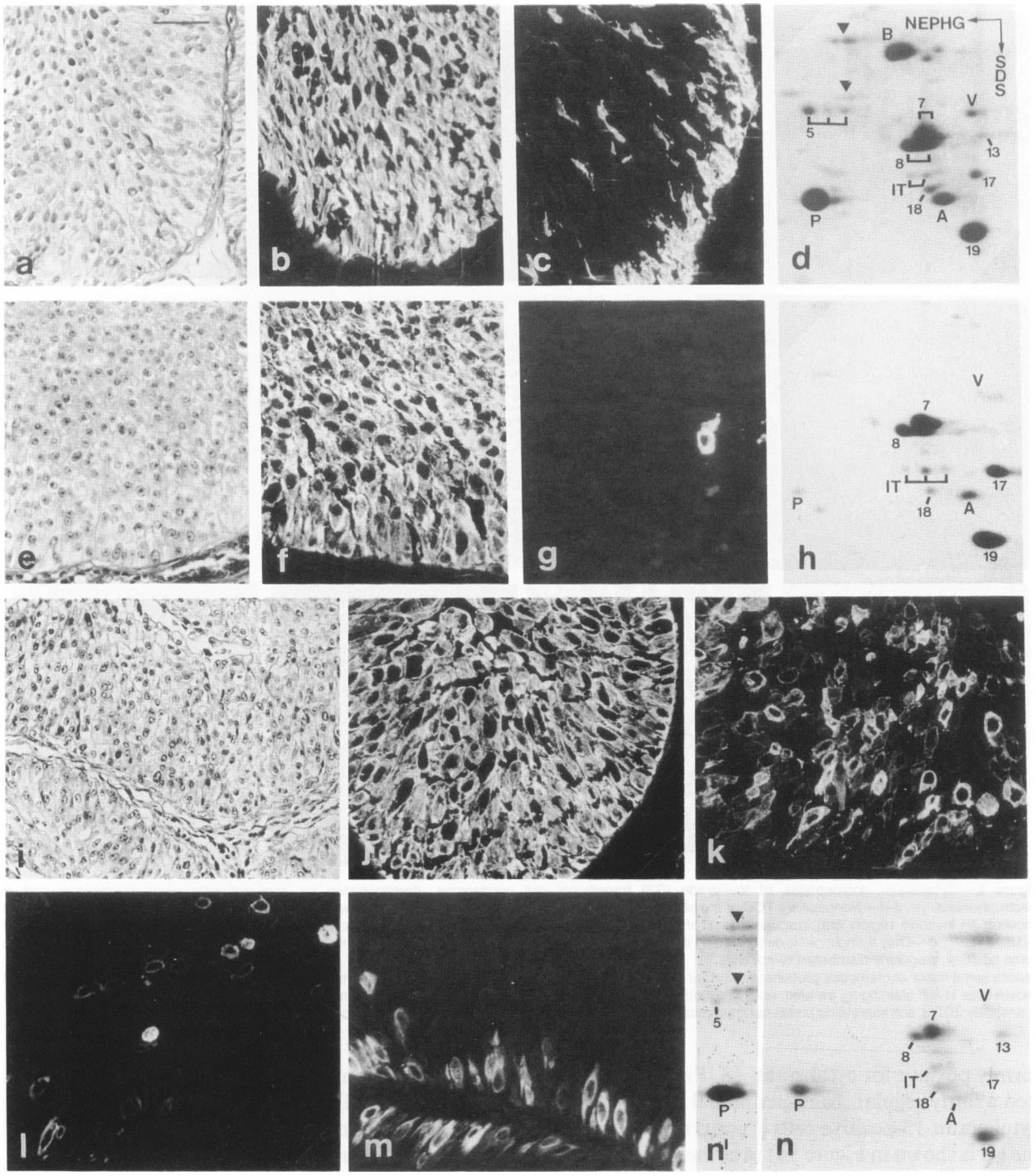


Figure 5—Cytokeratin polypeptides of papillary TCC of the bladder, G2, visualized by immunofluorescence microscopy and gel electrophoresis. **a–d**—Case 6 after H & E staining of paraffin-embedded material (**a**), immunofluorescence microscopy for cytokeratins 18 (**b**; antibody CK-2) and 13 (**c**; antibody 1C7), and gel electrophoresis (**d**; Coomassie Blue staining). **e–h**—Case 7 after H & E staining (**e**; paraffin), immunostaining for cytokeratins 18 (**f**; antibody K_S18.174) and 13 (**g**; antibody 2D7), and gel electrophoresis (**h**; Coomassie Blue staining). **i–n**—Case 8 after H & E staining (**i**; paraffin), immunofluorescence staining for cytokeratins 18 (**j**; antibody K_S18.174), 13 (**k**; antibody 2D7) and 4 (**l**; antibody 6B10), after staining with antibody IVD3A9 (**m**), and by gel electrophoresis (**n**, Coomassie Blue staining; **n'**, subsequent silver staining of the "basic" portion of the gel, revealing trace amounts of cytokeratin 5). Note that the three morphologically similar TCCs (compare **a**, **e** and **i**) reveal different cytokeratin patterns (compare **c**, **g** and **k**, and **d**, **h** and **n**). For designations see also legend to Figure 3l. Bar (in **a**), 50 μm.

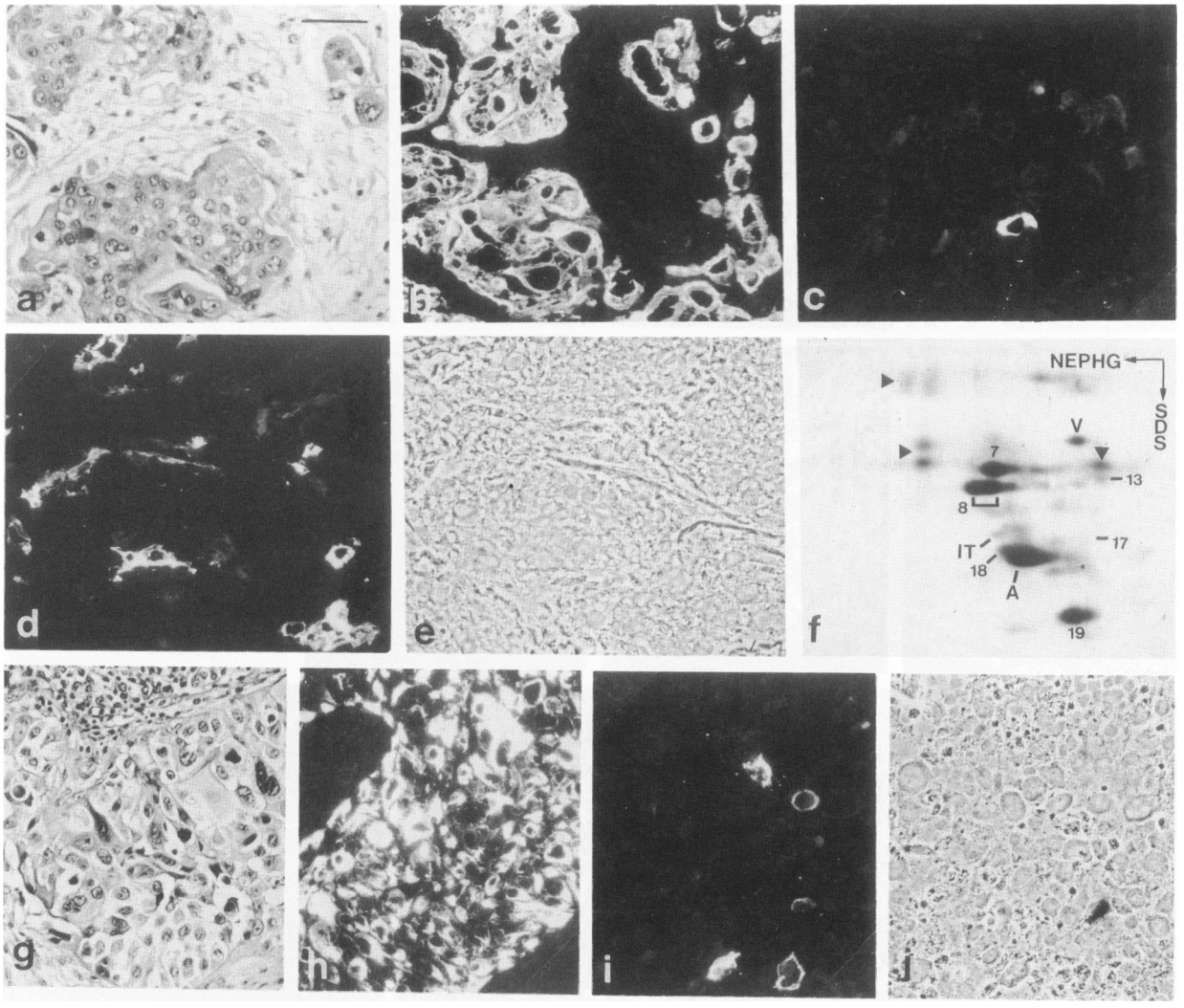


Figure 6—Cytokeratin polypeptides of low-grade (G3) transitional cell carcinomas, demonstrated by immunofluorescence microscopy and gel electrophoresis. **a–f**—Nonpapillary TCC of the bladder with tentacular invasion through all bladder wall layers (case 12). **a**—H & E staining (paraffin), showing an invasive region with irregular arrays of small tumor cell groups. **b**—Uniform immunofluorescence staining for cytokeratin 18 (antibody K_S18.174). **c**—Only a single cell is decorated by antibody 6B10 against cytokeratin 4. **d**—Immunostaining for cytokeratin 13 (antibody 1C7) revealing some positive, irregularly distributed tumor cells. **e**—Corresponding phase contrast picture to **d**. **f**—Gel electrophoresis (silver staining; arrowheads denote some minor contaminant proteins; for further designations see legend to Figure 3l). **g–j**—Lymph node metastasis of papillary TCC (case no. 14), shown after H & E staining (**g**, paraffin; note lymphatic tissue at the top) and immunofluorescence staining for cytokeratins 18 (**h**, antibody K_S18.174) and 13 (**i**, antibody 2D7; **j**, corresponding phase contrast micrograph). Note that in **i** only a few scattered tumor cells are positive. Bar (in **a**), 50 μ.

formly positive for cytokeratin 18 (Figure 5b), exhibited a fairly regular, basal-peripheral arrangement of cytokeratin 13-positive cells ("pattern B;" Figure 5c; the gel is shown in Figure 5d). Another G2 tumor pattern was characterized by prominent amounts of cytokeratin 17 in addition to the simple-epithelium-type cytokeratins, with only minor amounts of cytokeratin 13 ("pattern C;" Figure 5e–h). Three further cases with grade 2, 1 with areas of G3, which again were uniformly positive for cytokeratin 18 (Figure 5i, j), showed a considerable number of cytokeratin-13-positive cells in an apparently random distribution (Figure 5k), but relatively low amounts of this cytokeratin on gel electrophoresis (Figure 5n; "pattern D"). Heterogenous staining was observed with antibodies 6B10 (Figure 5l) and IVD3A9, the latter showing a conspicuous selectivity for certain basal cells in 2 of these cases (Figure 5m).

Poorly differentiated TCCs (G3) differed from G1 and G2 tumors by greatly reduced levels, or absence, of cytokeratin 13 and other stratification-related cytokeratins, so that the simple-epithelium-type cytokeratins were the predominant cytoskeletal proteins (Table 1). However, most of the G3 carcinomas still contained a few isolated cells positive for cytokeratin 13 and/or cytokeratin 4 (examples showing a primary nonpapillary tumor and a lymph node metastasis are shown in Figure 6a–f and g–j). In this pattern ("E")

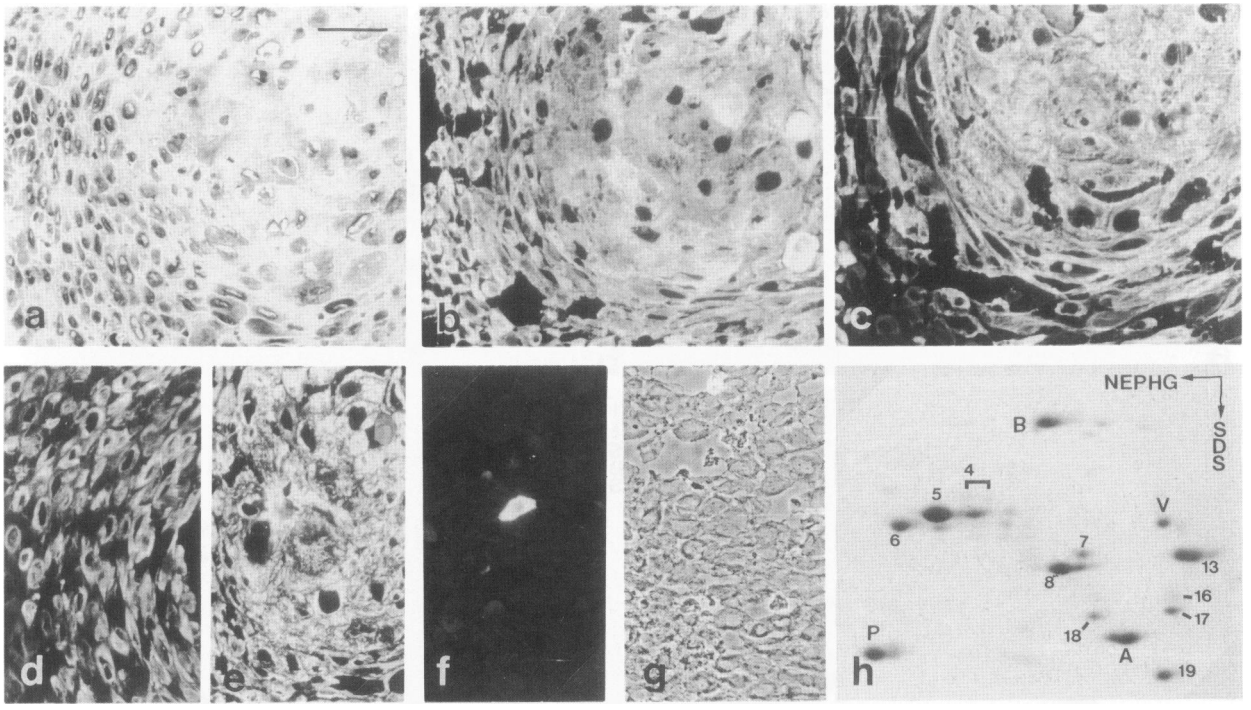


Figure 7—Cytokeratins of bladder TCC with squamous metaplasia (G2/3; case 20) shown by various methods. **a**—H & E staining of cryostat section of frozen material corresponding to the immunostained sections. **b–g**—Immunofluorescence microscopy on frozen sections with antibodies K_S18.174 against cytokeratin 18 (**b**), 6B10 against cytokeratin 4 (**c**; both showing regions of squamous metaplasia), IVD3A9 (**d**, showing a "nonsquamous" region; **e**, squamous region), and K_k8.60 against cytokeratins 10/11 that, in the region shown here, decorates a single tumor cell in a "nonsquamous" portion (**f**; **g**, corresponding phase contrast micrograph). **h**—Gel electrophoretic pattern showing a high proportion of the stratified-epithelium-type cytokeratins 5 and 6 (Coomassie Blue staining). This gel represents a tumor region with little squamous characteristics; in markedly squamous regions (see **b**, **c**, **e**), the gel pattern obtained was essentially similar, with the exception of a further reduced amount of cytokeratins 7 and 17 (not shown). For designations see legend to Figure 3l. Bar (in **a**), 50 μ.

gel electrophoresis revealed cytokeratin 13 either in only trace amounts (Figure 6f), or this protein was not detectable at all, whereas cytokeratin 17 was usually detected as a minor component (Table 1).

In TCCs with squamous differentiation marked changes of the cytokeratin pattern were found, notably a relative increase of stratification-related cytokeratins (Table 1). For example, in the TCC with squamous metaplasia shown in Figure 7, all regions were immunocytochemically positive for cytokeratin 18, regardless of the specific histologic appearance (Figure 7b). A similar, rather uniform immunostaining was produced not only by antibodies against cytokeratins 8 and 19 but also by antibodies against cytokeratins 13 (not shown), 4 (6B10; Figure 7c) and with antibody IVD3A9 (Figure 7d, e). In contrast, antibody K_k8.60 against cytokeratins 10/11 reacted with only a few single cells (Figure 7f, g). The gel electrophoretic pattern of this tumor was characterized by a high cytokeratin complexity, with prominent amounts of the stratified-epithelial cytokeratins 5 and 6 (Figure 7h; "pattern F"). In another tumor with focal squamous areas (noncornifying), conspicuous scattered and clustered cells positive with antibody K_k8.60 against cytokeratins 10/11 were noted (case 21 of Table 1).

A case of noncornifying squamous cell carcinoma of the bladder lacking morphologic features of transitional cells exhibited the most prominent stratified-epithelial cytokeratin characteristics among all tumors of this study and also showed a considerable reduction of simple-epithelial cytokeratins (case 22 of Table 1; "pattern G").

Bladder Carcinoma-Derived Cell Lines

Two basic patterns of IF protein expression could be distinguished by gel electrophoresis. In RT-112 and RT-4 cells cytokeratins were the only constituents detected and displayed a relatively complex pattern. In RT-112 cells IF polypeptides 7, 8, 13, 17, 18, and 19 were prominent components (Figure 8a), with the basic cytokeratin 5 as a minor component (Figure 8a, insert). A subclone of this line also revealed trace amounts of cytokeratins 4 and 6. In RT-4 cells, however, cytokeratins 7, 8, 17, 18, and 19 were also major proteins but cytokeratin 13 was identified only as a trace component (Figure 8b); in these cells cytokeratins 1–6 could not be detected by gel electrophoresis (Figure 8b, insert).

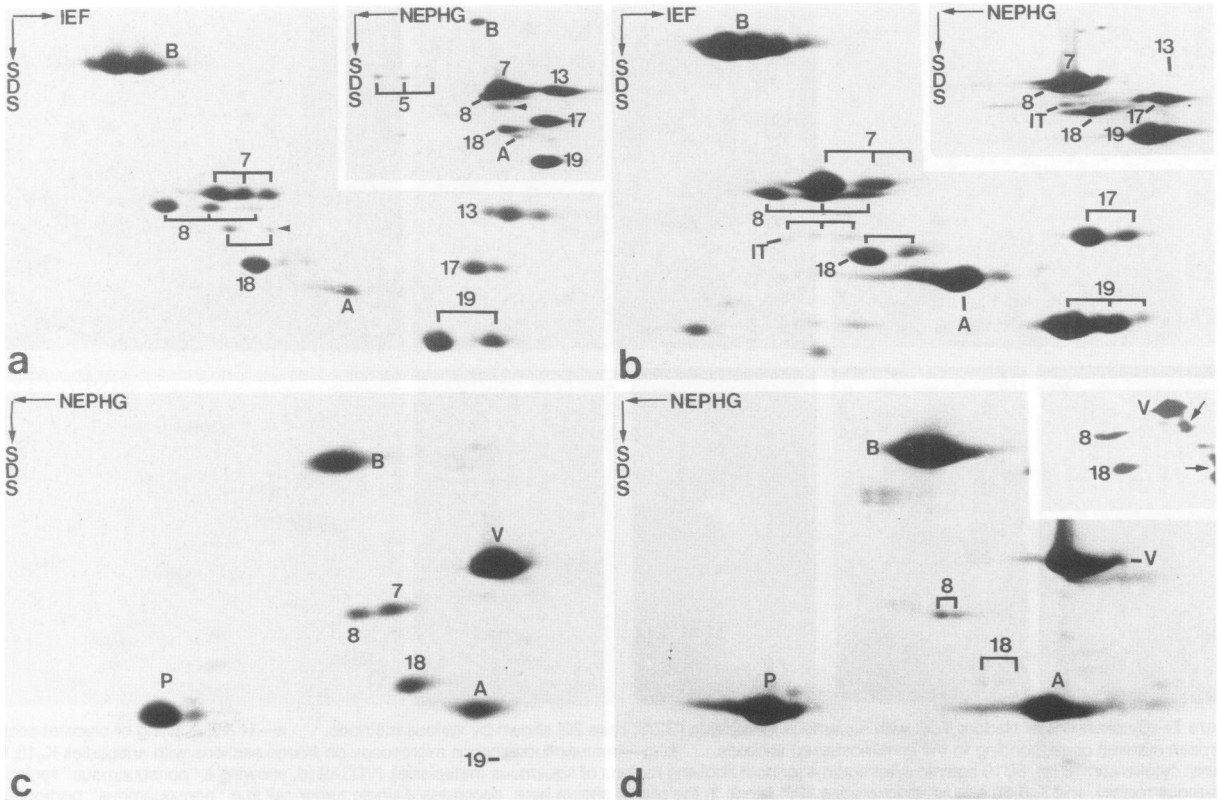


Figure 8—Two-dimensional gel electrophoresis of cytoskeletal proteins of cultured human bladder carcinoma cell lines, using either isoelectric focusing (IEF; **a, b**) or NEPHG electrophoresis (inserts in **a** and **b**; **c, d**). **a**—RT-112 cells (Coomassie Blue staining; insert, autoradiogram of ^{35}S-methionine-labeled cells; the unlabeled bracket and the arrowheads denote a degradation product of cytokeratin 8). Note that cytokeratin 5 is only a minor component (insert), whereas the trace amounts of cytokeratins 4 and 6 are not seen at this loading and exposure time. **b**—RT-4 cells (Coomassie Blue staining; insert, autoradiogram). Note that cytokeratin 13 is only expressed in trace amounts (insert); note also IT protein. **c**—T-24 cells (Coomassie Blue staining) expressing only the four simple-epithelial cytokeratins and a relatively large amount of vimentin (V). **d**—EJ cells (Coomassie Blue staining; insert, autoradiogram of labelled cells) containing large amounts of vimentin (V; arrows, degradation products of vimentin) together with small amounts of cytokeratins 8 and 18. A, endogenous actin (**a**) or α-actin from rabbit skeletal muscle added as reference protein (**b–d**).

In cytoskeletons of T-24 and EJ cells, both kinds of IF protein, cytokeratins and vimentin, were found, and in both cell lines, vimentin was a major component. In T-24 cells, cytokeratins 7, 8, and 18 were identified in considerable amounts whereas cytokeratins 17 and 19 were seen only as trace components and with very sensitive techniques (Figure 8c). Relatively small amounts of cytokeratins 8 and 18, as compared with the abundance of vimentin present, were detected as the only cytokeratins in EJ cells (Figure 8d).

Using immunofluorescence microscopy with a broad panel of antibodies specific for individual polypeptides it was possible to relate the biochemical findings to the cellular distribution of the IF proteins expressed in these cell lines. Again, the 2 basic patterns of synthesis of IFs were recognized. In RT-112 cells, guinea pig antibodies to cytokeratins 8 and 18 as well as monoclonal cytokeratin-18 antibodies such as K_S18.18 decorated a dense cytoplasmic fibril meshwork (Figure 9a); similar immunostaining was observed with the other antibodies specific for simple-epithelium-type cytokeratins, eg, K_S18.174, K_S19.1, CK-7, and K_S8.1.42 (not shown). Monoclonal antibody 1C7 specific for cytokeratin 13 stained most RT-112 cells but with different intensities (Figure 9b) whereas antibody 6B10 could detect cytokeratin 4 in a small proportion of the cells only (Figure 9c). RT-112 cells were negative for vimentin with all vimentin antibodies listed in the Materials and Methods sections (Figure 9d).

RT-4 cells showed a similar cellular distribution of cytokeratins (Figure 9e), with the exception of cytokeratin 13, which was detected here in a minority of the cells only (Figure 9f) corresponding to the small amount of gel electrophoretically detectable protein (see above). In this cell line cytokeratin 4 was detected in very few cells only (Figure 9g) and tests for vimentin were completely negative (Figure 9h).

In T-24 cells a homogeneous fibrillar staining was obtained with antibodies against cytokeratins 18 (eg, K_S18.174 in Figure 9i) and 8 (K_S8.1.42; data not

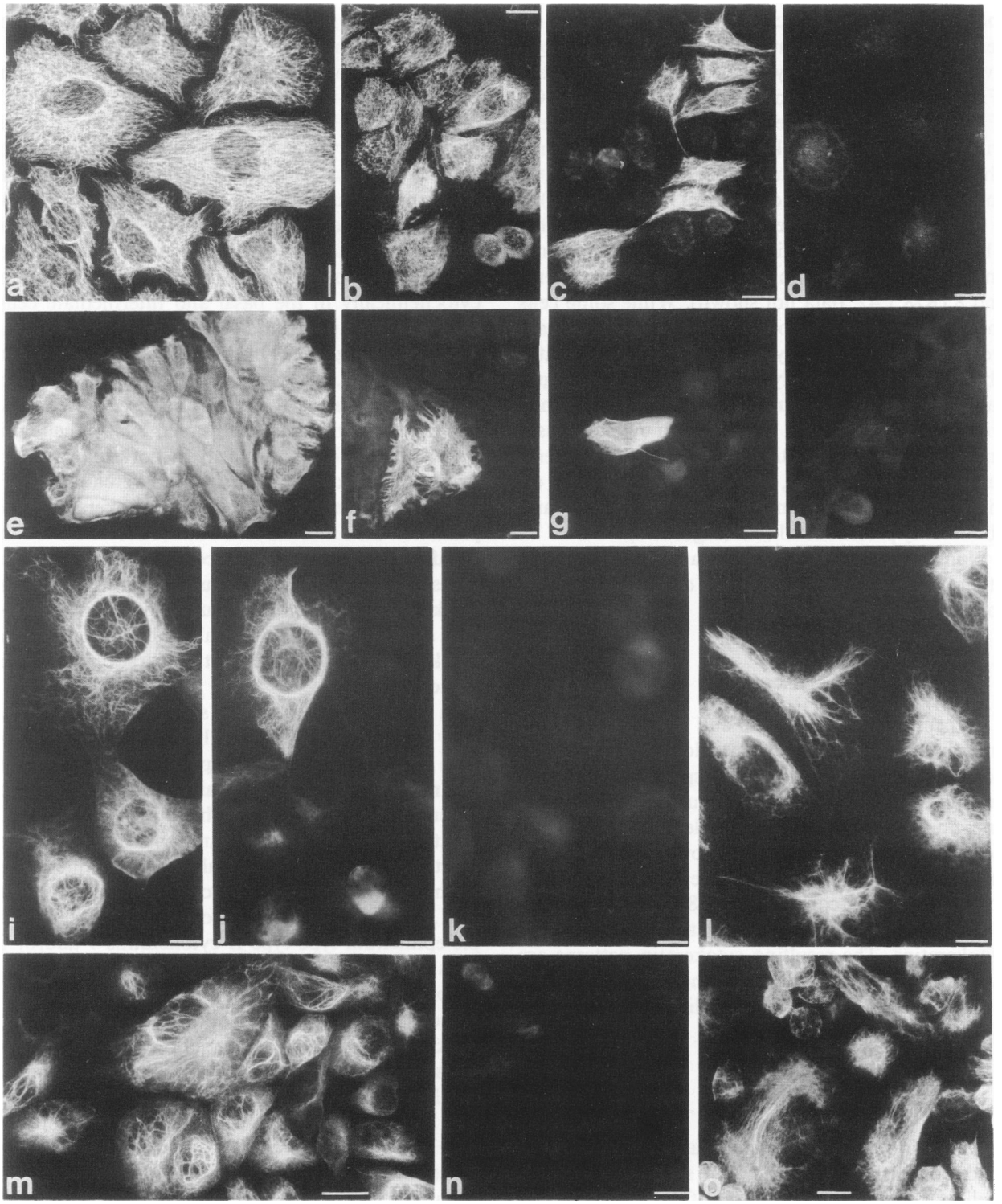


Figure 9—Immunofluorescence microscopy of human bladder carcinoma cell cultures using antibodies against cytokeratins and vimentin. **a–d**—RT-112 cells, exhibiting uniform fibrillar staining with antibody K_S18.18 against cytokeratin 18 (**a**), staining for cytokeratin 13, showing cell-to-cell differences of intensity (**b**; antibody 1C7), positive staining for cytokeratin 4 in only a few cells (**c**; antibody 6B10) and absence of staining with vimentin antibodies (**d**; guinea pig antibodies). **e–h**—RT-4 cells, showing uniform fibrillar staining of cytokeratin 18 (**e**; antibody K_S18.174) whereas cytokeratins 13 (**f**; antibody 1C7) and 4 (**g**; antibody 6B10) are detected only in very few cells; the reaction for vimentin is negative (**h**; guinea pig antibodies). **i–l**—T-24 cells are uniformly positive for cytokeratin 18 (**l**; K_S18.174) whereas antibody K_S19.1 (**j**) stains only some cells, either with an extended fibrillar pattern (top) or in a dense paranuclear aggregate (bottom). These cells are negative for cytokeratin 13 (**k**; antibody 1C7) but strongly positive for vimentin (**l**; guinea pig antibodies). **m–o**—EJ cells, showing positive reaction of cytokeratin 18 (**m**; antibody K_S18.174), although with marked cell-to-cell differences. These cells are negative for cytokeratin 13 (**n**; antibody 1C7) and uniformly positive for vimentin (**o**; guinea pig antibodies). Bars, 20 μ.

shown) whereas monoclonal antibody K_S19.1 revealed a heterogenous pattern, showing strongly positive cells next to weakly stained and negative ones (Figure 9j). Antibodies to cytokeratin 13 such as 1C7 (Figure 9k) were negative on these cells, in agreement with the absence of cytokeratin 13 in the gel electrophoretic analyses (Figure 8c). However, vimentin could be identified in all cells of this line (Figure 9l).

Cells of the EJ subline used in this study revealed a heterogenous cell staining pattern with monoclonal antibodies to cytokeratin 18 (K_S18.174 in Figure 9m) and 8 (K_S8.1.42; data not shown). While some cells showed an extensive positively stained fibril meshwork, others showed only a few decorated cytokeratin fibrils or small positively stained perinuclear aggregates (Figure 9m). A similar result also was obtained with guinea pig antisera reactive with cytokeratins 8 and 18 (not shown). Antibodies specific for cytokeratins 4, 7, 13 (Figure 9n), and 19 were all negative on these cells but vimentin IFs were consistently and abundantly present in all cells (Figure 9o).

None of these 4 cell lines appeared to contain any other IF protein, ie, GFP, desmin, or one of the 3 neurofilament polypeptides.

Discussion

Our immunocytochemical and gel electrophoretic analysis of cytokeratin patterns allow one to compare normal urothelium with a broad spectrum of urothelium-derived carcinomas and cultured carcinoma cell lines and to detect cell-type complexity and heterogeneity. Our results show that urothelial tumors are characterized by a basic, simple epithelium-type cytokeratin pattern (components 7, 8, 18, and 19) modulated by the addition of certain stratification-related cytokeratins. The resulting cytokeratin patterns are typical of certain subtypes of urothelial carcinomas in a way related to the morphologic differentiation of the specific tumors.

Normal Urothelium

Our results on normal urothelium confirm and extend our earlier studies[37,41,51,61] showing that urothelium contains large amounts of the simple-epithelium-type cytokeratins 7, 8, 18, and 19 and of the stratification-related cytokeratin 13, accompanied by smaller proportions of cytokeratin 5 and occasional traces of cytokeratins 4 and 17. (The amounts of the latter are so low that they have escaped detection in most previous analyses.) These biochemical data are in agreement with the concept of the urothelium as true stratified epithelium, albeit with a special histologic architecture.

Our immunolocalization data show that all 4 simple-epithelium-type cytokeratins are expressed in all cell layers of the urothelium, in agreement with previous studies.[51,56,62,69,84,85] The failure of certain monoclonal antibodies against cytokeratin 18 to stain the basal and intermediate cell layers[53,55] is probably due to cell-type specific epitope masking.[51,85] The prominent stratification-related cytokeratin of the urothelium, ie, component 13, has been localized immunocytochemically in the basal and intermediate cells, whereas the superficial cells have consistently been found to be negative. This is in contrast to a previous report[76] (which, however, may have included areas of squamous metaplasia) and surprising because in several noncornifying, stratified squamous epithelia this cytokeratin is found primarily, if not exclusively, in the suprabasal cells progressing in differentiation.[76,86] The alternative explanation that cytokeratin 13 is present in the umbrella cells but not accessible for antibodies (for "masking" of certain cytokeratin epitopes in umbrella cells of rat urothelium see[33]) seems unlikely because negative results have been obtained with all 3 different monoclonal antibodies against this cytokeratin and with different preparation and staining techniques.

In noncornifying, stratified squamous epithelia, the type II cytokeratin 4 is usually coexpressed with the type I cytokeratin 13, the 2 representing a cytokeratin "pair" characteristic of a certain pathway of epithelial differentiation.[40,86,87] In urothelium, however, cytokeratin 4 is found in trace amounts only and is restricted to a few scattered basal cells. Consequently, in the majority of the urothelial cells the type I cytokeratin 13 must form heterotypic complexes with the simple-epithelium-type type II cytokeratins 7 and/or 8. This is possible in view of the results of studies of *in vitro* reconstitution experiments showing that, in principle, each type I cytokeratin can form heterotypic pairs with each member of the type II cytokeratin subfamily.[78] Our observation that the primitive urothelium lining the renal papillae, which consists of 2–3 layers and lacks typical umbrella cells, contains only few cells positive for cytokeratin 13, which are mostly located in the basal layer and probably foci of beginning urothelial differentiation, is particularly interesting.

In addition to the cytokeratins mentioned above, small amounts of the stratified epithelium-type cytokeratins 5 and 17 occur in urothelium, and probably these proteins are responsible for the positive reactions of antibodies KA1 and IVD3A9 with certain basal layer cells. A staining pattern confined to basal

urothelial cells also has been described by Summerhayes and Chen[57] using an antibody against a murine M_r52,000 keratin that, on the basis of its cell type distribution, appears to be a stratification-related cytokeratin. The molecular basis for the immunostaining of umbrella cells with monoclonal antibody AE-2, believed to react with stratification-related cytokeratins,[38,62] remains unclear.

These observations indicate that in urothelium the expression of stratification-related cytokeratins does not follow the vertical increase pattern described for such cytokeratins in many stratified epithelia[37-40,76,77,86,87] but that, inversely, in the urothelium it is the basal and intermediate cell layers that exhibit molecular features of squamous cell differentiation. This may be related to the potential of this epithelium to develop squamous metaplasia.[8-13] A similar restriction of stratification-related cytokeratins to certain basal cells has been described in some complex, nonstratified epithelia, such as in the tracheal and bronchial linings.[88] Because the basal cell layer of the urothelium is commonly assumed to represent the proliferative compartment,[1,2,7,12] it is difficult to explain why in this tissue, in contrast to other stratified epithelia, it is the basal cell layer that displays a cytokeratin pattern more complex than that of the uppermost cell layer, ie, the umbrella cells. In this context, however, it is important to remember that it is the umbrella-cell layer that has typical features of a polarized epithelial cell, such as a well developed junctional complex with a typical zonula occludens, an organization similar to that of polar simple epithelia.

The presence of cytokeratins 7, 8, 18, and 19 in all urothelial cell layers also explains the known intense reaction of antibodies to the "tissue polypeptide antigen" (TPA),[89,90] a widely used serodiagnostic tumor marker that has been identified recently[91] as fragments of cytokeratin 8, with all urothelial cell layers and urothelial carcinomas.

Transitional Epithelium-Derived Carcinomas

Our analyses of cytokeratin expression in a broad spectrum of transitional cell-derived carcinomas have led to 3 main conclusions: 1) Most pure TCCs retain cytoskeletal features of the urothelium, although to a variable extent. 2) Different types of TCCs can be distinguished by their cytokeratin patterns, as we have suggested previously.[51] 3) There is a correlation of the cytokeratin pattern with the grade of malignancy of the specific TCC. 4) The presence of squamous metaplasia in such tumors seems to be correlated with higher proportions of stratification-related cytokeratins, including some (eg, components 4, 6, 14, and 16) either not found or found rarely in the normal tissue and in typical TCCs.

In low-grade (G1, G1/2) TCCs, the malignant tissue essentially maintains the cytokeratin pattern of normal urothelium ("pattern A", Table 1; see also[53]), in particular that of the basal-intermediate compartment, including prominent amounts of cytokeratin 13. Umbrella-celllike superficial cells are seen in some of these cases and resemble normal urothelial umbrella cells in their relatively strong staining for cytokeratins 8 and 18 and an at least partial negativity for cytokeratin 13. Interestingly, the cell-type-specific masking in lower urothelial layers of certain cytokeratin epitopes such as that of antibody RGE-53 appears to be maintained in such low-grade TCCs.[53,55]

In contrast, G2 and G3 tumors are characterized by much lower levels of cytokeratin 13 as well as other minor changes, whereas the expression of large amounts of simple-epithelium-type cytokeratins is maintained. While G2 TCCs, which show some variation in their basic cytokeratin patterns, despite their similar morphology, usually contain a certain proportion of cells positive for cytokeratin 13, this cytokeratin is greatly reduced or even absent in poorly differentiated G3 TCCs. This overall correlation between the level of expression of cytokeratin 13 and the grade of malignancy as judged from morphologic criteria may represent a general loss of urothelial differentiation features in G3 tumors. Nevertheless, the observation of a few cells positive for cytokeratin 13 in most G3 carcinomas, including metastases, may be of diagnostic significance in cases of poorly differentiated metastatic tumors of unknown primary origin. In all cases examined, comparisons of primary tumors and metastases[50] have shown a high degree of stability of cytokeratin pattern expression. However, a few G3 tumors contain only simple-epithelium-type cytokeratins, together with some minor amounts of cytokeratin 17, and thus are not distinguishable in their cytokeratin pattern from certain adenocarcinomas such as those of breast, pancreas, gall bladder, and uterine cervix.[37,41,61,67,86]

The presence of focal squamous metaplasia defines a morphologically well-known group of variant TCCs (10–20%).[8-14] Such tumors display cytokeratin patterns different from those of pure TCCs, characterized not only by an increase in certain stratified-epithelial cytokeratins such as components 4, 5, and 17 but also by the apparently new expression of cytokeratins 6 and 16, which are prominent components of squamous cell carcinomas of other organs.[37,39-41,50,61,67,88] Most unexpected is the expression in some tumors of this category of cytokeratins 10/11 usually considered

typical for terminal differentiation, notably in the epidermis.[37,41,61,77,87,92] Immunocytochemically, we have found cytokeratins 10/11 in scattered cells or cell clusters in 3 cases, 2 of which exhibited squamous metaplasia (for possibly related observations with other, yet unclassified antigens associated with epithelial stratification in certain squamous forms of human and animal bladder tumors see[54,56,60]). In summary, we can conclude that, despite the increase in cytokeratins of the stratified-epithelial group, TCCs with foci of squamous metaplasia still express relatively high levels of cytokeratins 7, 8, 13, 18, and 19, reflecting their urothelial origin. Their cytokeratin patterns are thus quite different from those of most types of squamous cell carcinomas of other organs,[37–41,61,67,76,86,88] including the single case of pure squamous cell carcinoma of the bladder examined in this study (Table 1).

Bladder Carcinoma Cell Lines

Our characterization of the IF cytoskeletons of 4 permanent human cell lines (RT-112, RT-4, T-24, and EJ), all of which have been reported to be derived from TCCs of the urinary bladder,[19–25] clearly demonstrate their epithelial origin and in this respect are in agreement with some previous reports.[30,53,63] However, our results on the coexpression of cytokeratins and vimentin in these cell lines are partly at variance with those of other groups. Using both immunofluorescence microscopy and gel electrophoresis we found that only T-24 and EJ but not RT-112 and RT-4 cells synthesize vimentin, whereas Ramaekers et al[53] describe vimentin immunoreactivity in their RT-4 cell cultures and Wu et al[63] report the observation of vimentin in occasional RT-112 cells. Because the advent of vimentin IFs in cytokeratin-expressing epithelial cells growing in culture is a widespread phenomenon[30,42,47,93–95] and is obviously influenced by the growth media and conditions,[30,94,95] it is possible that the same original cell line may start to form vimentin IFs under the specific growth conditions of one laboratory but not under other conditions.

The cytokeratin patterns of 3 of the 4 cell lines studied show a remarkably good correlation with those of TCCs of the respective grade. The pattern of RT-112 cells fits the "A" pattern typical of G1 and G1/G2 tumors, that of RT-4 cells fits the "C" pattern found in certain G2 tumors. Indeed, both these lines have been reported to be derived from well-differentiated TCCs. The cytokeratin complement of T-24 cells, which are reportedly derived from a poorly differentiated tumor, resembles the "E" pattern of G3 TCCs (Table 1). This suggests a high stability of the expression of the cytokeratin pattern of a given carcinoma cell during *in vitro* culture. We conclude that these 3 cell lines faithfully express TCC-typical cytoskeletal proteins and therefore provide suitable model cell systems for studies of TCC and/or urothelial differentiation properties *in vitro*.

Surprisingly, our IF protein analyses have shown that our EJ cell cultures, which have been reported to be identical with T-24 cells by HLA-typing and isoenzyme analysis,[25] differ from T-24 cells by the absence of cytokeratins 7 and 17 (the recloned EJ subline used in the present study also lacks cytokeratin 19, previously reported to be present in a less well defined EJ culture[41]). At present we cannot conclude whether EJ cells have changed their cytokeratin pattern during culturing, or whether they are derived from a tumor cell different from the T-24 precursor cell(s). Clearly, however, our findings indicate that a cell line with this cytoskeletal protein pattern may not provide an adequate model of urothelial differentiation. In addition, it has to be noted that other cytoskeletal markers positive in EJ and T-24 cells, such as the antigen recognized by monoclonal antibody H10-197, are missing in certain cell lines of demonstrated urothelial character (RT-112, RT-4) but are present in a many other carcinoma cell lines derived from non-urothelial tissues.[96]

Cell Type Heterogeneity

Another important and unexpected result of our immunocytochemical studies is the observation of cell type heterogeneities with respect to reactions of certain cytokeratins in normal urothelium, in the diverse tumors, and in the cell cultures. This heterogeneity, which has also been noted in some of the cloned cell lines, notably for cytokeratins 4 and 13 in RT-112 and RT-4 cells and for cytokeratin 19 in T-24 cells, seems to be an intrinsic principle of divergence as it appears again and again in clonally selected sublines. By karyotype analyses, morphologic criteria and growth characteristics cell-type heterogeneity has previously been noted in several urothelium-derived cell lines, including T-24[20] and EJ.[23] At present we cannot say whether such heterogeneities, in tissues as in cultures, reflect spontaneous "decision" differences in the biosynthetic program of individual cells or are due to microenvironmental influences as, for example, local differences of certain hormones and vitamin A, which are known to have effects on the expression of certain cytokeratins.[92,94,95,97] It will be important to find out whether a relationship exists between these cytoskeletal cell type heterogeneities, in cultured cell lines and in normal urothelium, to the cell-type heterogeneities observed in tumor tissues (see Results sec-

tion and[55,56]), and how this may be related to the cell-type heterogeneities in tumor behavior that may give rise to more or less invasive and metastatic derivatives (for discussion see[7-18]).

Conclusions and Perspectives

Our analysis of the distribution of cytokeratins in the urothelium has emphasized the unique organization of this stratified epithelium. While in all other multilayered epithelia so far studied the cytokeratins typical of vertical differentiation are expressed in suprabasal cell layers, the urothelium shows the inverse situation: As judged from immunocytochemistry, cytokeratin 13, which is abundantly present in this tissue, appears to be confined to basal and intermediate cell layers and absent from superficial cells, and the cells positively immunostained for other stratification-related cytokeratins such as polypeptide 4 are also exclusively found in the basal compartment. This inversion of layer differentiation and the reported longevity of the umbrella cells (half-life time, 200 days or more; for reviews see[1,2,7,12]) raise some fundamental questions about the mechanism of vertical differentiation and tumor formation in this tissue. It is indeed difficult to reconcile the profound and sharply demarcated differences in cytoskeletal composition and in junction organization with the common view of the formation of umbrella cell by fusion of cells of the intermediate layer.[1,2,7,12] A derivation of the umbrella cells from basal cells would imply not only the selective halt of synthesis of cytokeratin 13 and the complete "disappearance" of this protein but also the *de novo* formation of a complete subapical junctional zone, with the entire zonula occludens, which would be a remarkable achievement for a terminally differentiated cell. Therefore, the data available, including the similarities of cytoskeletal composition and ultrastructure between the urothelial umbrella cells and the primitive urothelium of the renal papillae as well as the demonstrated mitotic potential of the umbrella cells,[1,2,7,12] should initiate some reconsideration of concepts of cell differentiation and carcinogenesis in the urothelium.

The cytoskeletal characteristics of most urothelial tumors, notably their cytokeratin composition, show similarities to those of the normal urothelium. Although the cytokeratin pattern can be subject to changes in the diverse neoplasias, these changes seem to be rather systematic and, on the whole, related to the state of differentiation of the specific tumor. Marked differences of cytokeratin expression exist between TCCs with and without regions of squamous metaplasia and between TCCs and squamous cell carcinomas (for the latter see also[56]). Our results point to a series of possible diagnostic applications such as in the identification of urothelial differentiation in morphologically unclear cases, especially in metastatic tumors, in the detailed grading and staging and in cytologic examinations of urinary sediments.[9,98] The distinction of 5 different cytokeratin patterns in morphologically pure TCCs should be a valuable additional criterion for a more subtle, nonmorphology-dependent TCC subtyping than is currently possible by conventional morphologic methods. Since the correlation between the morphologic classification of TCCs and their biologic behavior is still unsatisfactory, it would be of considerable clinical importance to clarify whether the specific TCC cytokeratin phenotypes could be correlated with certain invasive and metastatic properties.

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Note Added in Proof

A recent paper reported staining of 2 selective cytokeratin antibodies on normal and pathologic urothelium (Cintorino M, Del Vecchio MT, Bugnoli M, Petracca R, Leoncini P: Cytokeratin pattern in normal and pathological bladder urothelium: Immunohistochemical investigation using monoclonal antibodies. J Urol 1988, 139:428–432). In most aspects their data agree with ours.