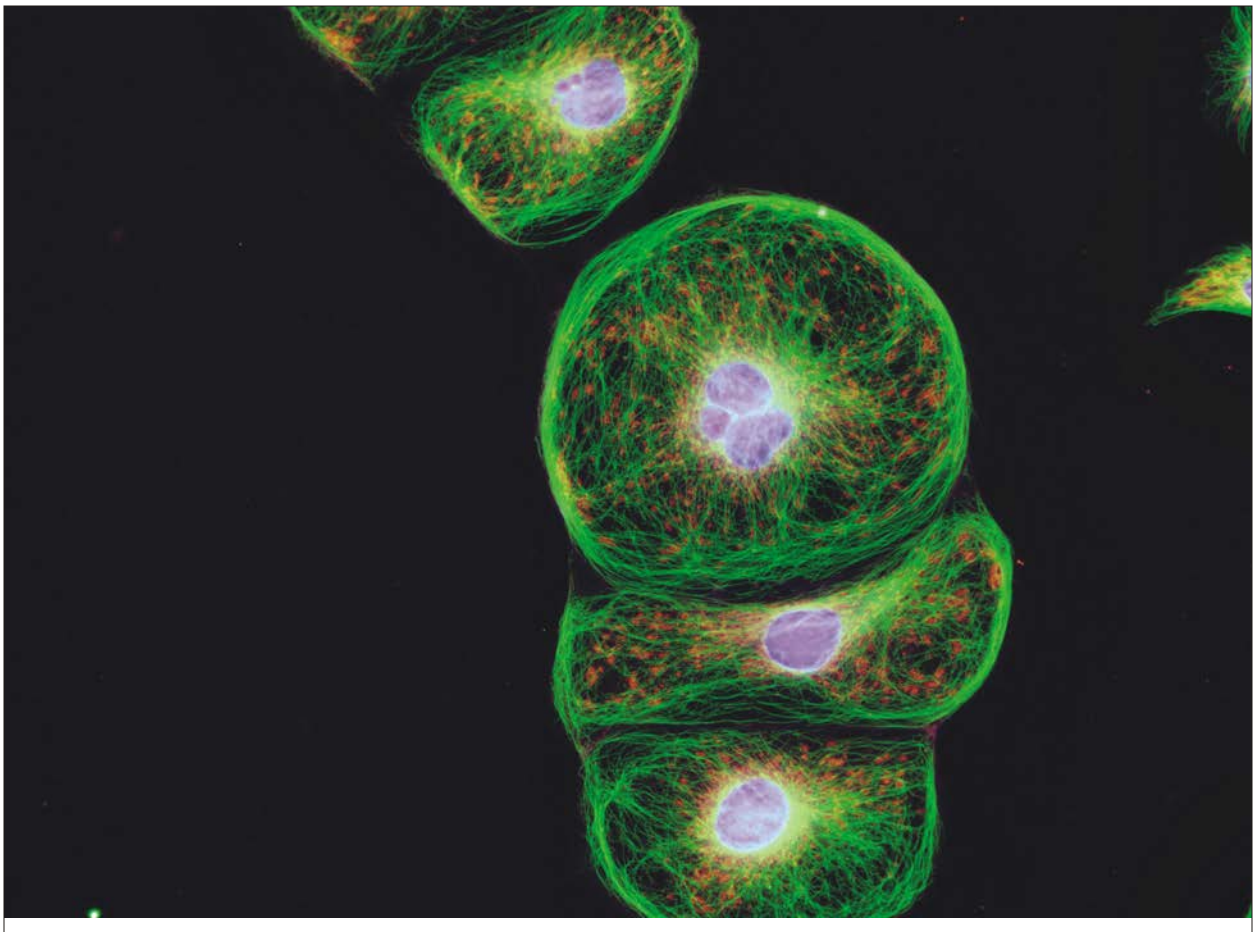

Asian Journal of Cell Biology

ISSN 1814-0068

Academic Journals Inc.

www.academicjournals.com

Asian Journal of Cell Biology 1 (1): 1-8, 2006
ISSN 1814-0068

The Cytokeratins of Urinary Bladder Epithelial Cells

Peter Veranič and Kristijan Jezernik
Institute of Cell Biology, Medical Faculty of Ljubljana,
1000 Ljubljana, Slovenia, EU

Abstract: Urothelium is a stratified transitional epithelium covering the luminal side of urinary bladder. The superficial urothelial cells have an amazing ability to change their diameter in order to accommodate to alternating volume of the urine in bladder. A dense cytokeratin network was found just below the apical membrane most likely to support the cells during stretching. A special type of vesicles (fusiform vesicles) represent the intracellular pool of apical membrane which can enlarge the apical surface when required. The trajectorial organisation of the cytokeratin network enables transport of vesicles through this network which still retains its mechanical supportive function. Formation of this cytokeratin network in superficial urothelial cells can be followed during embryonic development or through regeneration after urothelial destruction with cyclophosphamide. In healthy urothelium each layer of cells express its own profile of cytokeratins that can be used as markers of differentiation. This profile can be changed during neoplastic transformation of urothelial cells. Thus, the detection of certain cytokeratins is an important diagnostic tool for tracing tumour cells.

Key words: Cytokertatin structure, cytokeratin organisation, bladder regeneration, urothelial carcinoma

Structure and Function of Urothelium

Luminal side of a urinary bladder is covered by a stratified epithelium-urothelium composed of three types of cells including basal cells, intermediate cells and superficial cells, which are because of their shape, also called umbrella cells. Small basal cells (10 μm) lay attached to the basement lamina. They have the highest proliferation potential and serve as precursors for upper layers of urothelial cells. Intermediate cells are larger (10-25 μm) and are arranged in one to several layers. Some of this cells are supposed to have thin connections to the basement lamina (Hicks,1975). On top of intermediate cells and therefore in contact with urine are large polyhedral umbrella cells (25-250 μm) which are mainly responsible for the maintaining of the blood-urine barrier. The composition of urine is very different from blood by osmolality ranging from 50 to 1000 m osmol kg^{-1}, pH around 4.5 and high concentration of urea and potential carcinogens (Truschel *et al*., 1999) The prevention of the diffusion of urine components through mucosa into other tissues is one of the main functions of the urothelium (Apodaca, 2004). The apical membrane and the tight junctions of umbrella cells are the crucial components of the blood-urine barrier. Depending on interspecies variability 70-90% of the apical membrane of umbrella cells is covered with polygonal concave plaques containing characteristic proteins-uroplakins, which are mainly inserted in the extracellular leaflet of the membrane. The plaque

Corresponding Author: Peter Veranič, Institute of Cell Biology, Medical Faculty of Ljubljana, 1000 Ljubljana, Slovenia, EU Tel: 386 1 543 76 80 Fax: 386 1 543 76 81

regions are thicker (12 nm) and because of uroplakins asymmetric in comparison to the remaining normal membrane (8 nm) which surrounds individual plaques and serves as hinges. The urothelial tight junctions are composed of 4-6 interconnecting strands and are supposed to be the most impermeable in mammalian body (Lewis and Diamond, 1976). Together with the apical membrane the tight junctions give the astonishing transepithelial resistance that can in rabbit be up to 75000 Ω cm^2.

The differentiated superficial cells of urinary bladder epithelium have an amassing ability to change their shape during the expansion and contraction cycles of the bladder. Such ability of superficial cells is enabled by two unique characteristics. The first one is a very dense cytoskeleton in their apical cytoplasm (Hicks *et al*., 1965; Staehelin *et al*.,1972) . Of the cytoskeletal elements in umbrella cells the cytokeratins were found to be the most prominent (Romih *et al*., 1999). The second characteristic is a mechanism for the enlargement of the asymmetric apical plasma membrane by a transfer of the membrane from the cytoplasmic pools known as discoid fusiform vesicles (Hicks, 1965; Staehelin *et al*., 1972; Robertson and Vergara,1980) .

Cytokeratin Physical Properties and Their Expression in Urothelium

The extreme ability of urothelial cells to expand (Baskin *et al*., 1994) requires a strong mechanical support to resist to such stretching forces. The best candidates for such task among cytoskeletal filaments are the cytokeratins, which major role is to protect cells against mechanical stress (Coulombe *et al*., 2000; Galou *et al*., 1997). The micromechanical property, which enable cytokeratins to perform their protective role, is first of all much larger elastic modul (G') compared to other cytoskeletal elements, for example G' for actin is 10 dynes cm^{-1} while for keratin it is 40-60 dynes cm^{-1}. It means that higher input of energy is required for deformation of the filaments. In contrast to other cytoskeletal filaments the elasticity of cytokeratins increases in response to strain. Another unique characteristic of the cytokeratins is their almost immediate recovery after yield which enable cells to regain their original shape when the strain is released (Coulombe *et al*., 2000). The mechanical properties of cytokeratins depend on their special structure. Cytokeratins consist of more than 20 isotypes of proteins that include types I (CK9-CK20) and types II (CK1-CK8). In all epithelial cells intermediate filaments are composed of at least one type I and one type II cytokeratin which form coiled-coil dimmers as heteropolymers. Regardless of the number of cytokeratins expressed in a given epithelial cell the ratio of type I to type II is always 1:1(Coulombe and Omary, 2002; Moll *et al*., 1982). Experiments using viscosimetry have shown that CK filaments formed from different subunits have different mechanical properties (Hofmann and Franke, 1997). The cytokeratin profile of an epithelial cell depends on special tasks of the tissue and on the level of differentiation. Thus, in urothelium different cytokeratin profiles can be detected in cells at different tissue layers. For example, cytokeratin 5 and 17 are characteristic for basal cells, cytokeratin 13 for basal and intermediate cells, while cytokeratin 20 is present exclusively in umbrella cells. On the other hand cytokeratin 7, 8, 18 and 19 are supposed to be expressed throughout all urothelial cell layers (Guelstein *et al*., 1993; Moll *et al*., 1990; Romih *et al*., 1998; Schaafsma *et al*.,1989; Southgate *et al*., 1999).

Organisation of Cytokeratins in Umbrella Cells

The ultrastructural studies from the middle sixties and seventies of the 20th century, predicted that the cytoplasmic filaments are associated with fusiform vesicles (Hicks, 1965; Minsky and Chlapowski, 1978) and also with plaques of asymmetric membrane at the apical plasma membrane of

umbrella cells (Staehelin *et al*., 1972). In previous study (Veranič and Jezernik, 2002) it has been revealed that cytokeratins 7 and 20 accumulate as a dense structure in the subapical region of umbrella cells. Yet, this cytokeratin structure should not hinder a very intensive traffic of the fusiform vesicles involved in alterations of the surface area of the apical membrane. Therefore, the cytokeratins have to be organised in a way to be both mechanically strong and also passable for fusiform vesicles. The studies with confocal laser microscopy and transmission electron microscopy revealed that the cytokeratin 7 and cytokeratin 20 compose a subapical network, which is constructed as an array of parallel trajectories pointing to the apical plasma membrane (Fig. 1). The double immunolabelling of the urothelial proteins uroplakins and cytokeratins proved the presence of fusiform vesicles within these

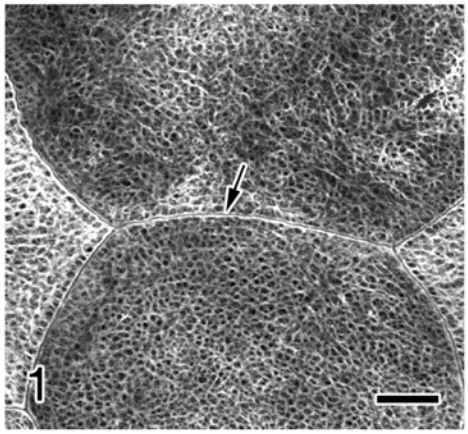

Fig. 1: Optical section of superficial urothelial cells represents the cytokeratin network in the subapical area of cells. Toward the lateral membrane, the cytokeratin network ends with a frame (↑), which is parallel to the frame of the neighbouring cell. Bar = 5 µm

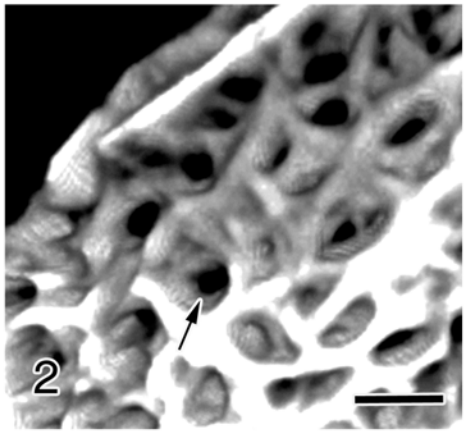

Fig. 2: The structure of the conical trajectories (↑) which penetrate through the layer of cytokeratins. The view from the cytoplasmic side. Bar = 1 µm

trajectories. Fusiform vesicles were shown to be accumulated in the trajectories and it is most likely that they are transported along the trajectories to the apical membrane during distension of the bladder and in the opposite direction during bladder shrinking. By 3D reconstruction of serial optical sections it was found that the trajectories are conically shaped with a smaller diameter pointing to the apical membrane and a larger diameter opening to the cytoplasm (Fig. 2). The mean diameters of the trajectories largely depend on bladder distension. In contracted bladder the mean diameter in the middle section of the network is less than 0.7 μm while in distended it excided 1 μm. We proposed that this enlargement of the trajectory diameter facilitates or even regulates the transport of fusiform vesicles to the apical membrane. Discovery of the subapical cytokeratin network elucidated the until now un-described supramolecular organisation of cytokeratins in the apical region of urothelial cells (Veranič and Jezernik, 2002)

The Formation of the Cytokeratin Trajectory Network in Umbrella Cells

In intermediate cells of urothelium the cytokeratins are organised as more or less individual filaments which are attached to desmosome plaque regions. The formation of trajectorial network takes part during final differentiation of superficial cells. Because of the long life span of urothelial cells, it is a very infrequent situation in a normal healthy urothelium to find an umbrella cell during development. The best models to study the formation of the cytokeratin organisation were found to be the developing bladder in the embryonic animals (not published yet) or regeneration of the urothelium after cell damage induced by cyclophosphamide (Veranič *et al*., 2004).

In mice the urothelium develops from the stage of urogenital sinus to completely differentiated urothelium during the last three to four days of embryonic life. It became evident in our recent experiments that from diffuse labelling of cytokeratin 7 the basic scaffold of the network become organised at the 16th embryonic day. The network is very densely packed and located close to the apical membrane. The cytokeratin 20 can be first detected at the 16th day as a very weak and diffuse labelling which is sporadically distributed in individual cells in urothelium. It is interesting that urine starts to accumulate in bladder on the same day as cytokeratins 20 appears first (Jezernik and Pipan, 1993). After that the cytokeratin 7 network became less compact in comparison to previous stages, while the quantity of cytokeratin 20 gradually increases until it reaches the labelling intensity and construction very similar to situation in adult mice.

In embryonic mice the development of trajectorial cytokeratin network is completed in just three days. Thus, for the more precise analysis of the network formation the induced regeneration after a large scale destruction of urothelium with cyclophosphamide was proved to be more suitable. Cyclophosphamide is a common cytostatic which metabolites, mainly acrolein and the induced synthesis of nitric oxide, cause necrotic destruction of urothelial cell (Jezernik *et al*., 2003; Oter *et al*., 2004). The regeneration of urothelium includes differentiation of cells from basal to superficial cells. At early stages of superficial cell differentiation cytokeratin 7 express filamentary organisation. After that cytokeratin 7 gradually reorganizes into a trajectorial network. Predominantly, the network organizes from the lateral rim toward the centre or as certain focal origins in the subapical cytoplasm. To some extent similar development of cytokeratins was found also in post-mitotic vulvar carcinoma derived cells A431. The network of cytokeratin 13 in this cells was demonstrated to develop exclusively from cell cortex by the aid of both actin filaments and microtubules (Windoffer and Leube, 2001). For cytokeratin 20 of umbrella cells it was found that

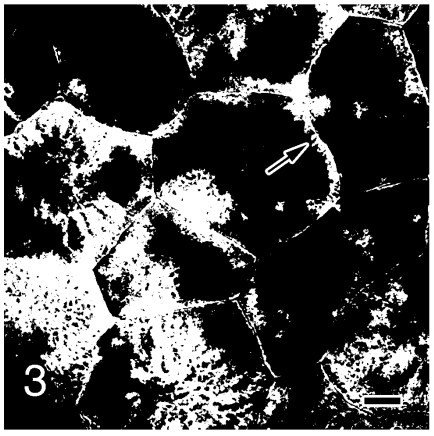

Fig. 3: 3 days after cyclophosphamide treatment the cytokeratin 7 is distributed at the lateral edge (↑) or focally in the central area of superficial cells. Bar = 10 μm

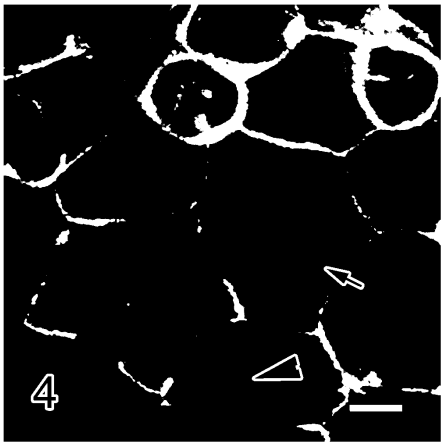

Fig. 4: 5 days after cyclophosphamide treatment the cytokeratin 20 labelling shows weak but continuous network in individual cells (↑), while in others the labelling is completely negative (▲). Bar = 10 μm

during network formation the labelling does not show any areas of origin, as found for cytokeratin 7, but a gradual increase of the labelling intensity and the number of cytokeratin 20 positive cells. Thus, it is predicted that cytokeratin 20 became incorporated into the pre-existing trajectorial cytokeratin network in which cytokeratin 7 is one of the components (Veranič *et al*., 2004) (Fig. 3 and 4).

In both embryonic development and regeneration after treatment with cyclophosphamide it became evident that cytokeratin 7 forms a template for the trajectorial network while cytokeratin 20 most likely influences the elasticity of the network, which enables cells to adjust to the periodical changes of urine volume in bladder. Disappearance of cytokeratin 20 from urothelium in patients with spinal chord injury, where the storage function of the bladder is disabled (Vaidyanathan *et al*., 2002), speaks in favour to this hypothesis.

Cytokeratins as Markers of Urothelial Pathology

Urothelium is subject to neoplastic transformations of various stage and grade of malignancy from benign hyperplasia to invasive-high grade urothelial carcinoma (also called transitional cell carcinoma) (Epstein *et al*., 1998). Besides histological changes of urothelial cells, one of the crucial hallmark for the neoplastic transformation is a change in expression of cytokeratins (Southgate *et al*., 1999). Some of the cytokeratins are retained in neoplastic cells or even become more expressed as for example the cytokeratin pair 8/18 which expression increases especially in invasive tumour cells bordering stroma (Schaafsma *et al*., 1990). Similarly cytokeratin 17, which is characteristic for basal cells of normal urothelium, in tumours it became expressed in all layers of urothelium (Guelstein *et al*., 1993). For other cytokeratins like cytokeratin 13 a reduced expression has been shown in invasive urothelial neoplasms (Schaafsma *et al*., 1989). On the other hand cytokeratin 14 which is not found in normal urothelium can be detected in some urothelial carcinomas (Moll *et al*., 1988). Cytokeratin 20 is a marker of umbrella cell differentiation. Also in tumours retaining cytokeratin 20, it is mainly considered as a marker showing good prognosis for the patient (Harnden *et al*., 1995).

Detection of cytokeratins is also important for distinguishing between metastatic carcinomas originating from different tissues i.e. urinary bladder, ovary, breast or lung. Namely treatment and prognosis largely depend on tissue where primary tumour developed. Usually expression of pairs of cytokeratins are examined as for example cytokeratin 7 and 20. For urothelium derived metastasis both cytokeratins are supposed to be positive, while carcinomas from lung, breast and ovary usually express cytokeratin 7 but are negative for cytokeratin 20 (Gown, 1999; Samaratunga and Khoo, 2004).

Not only immunolocalisation of cytokeratins in tissue is important as a diagnostic tool. Detection of partially degraded cytokeratins 8, 18 and 19 in urine, probably released from necrotic carcinoma cells, were proposed to become routine diagnostic procedure for detecting bladder cancer and for screening of its recurrence (Morita *et al*., 1997; Pariente *et al*., 1997).

Cytokeratins are intimately involved in both differentiation and in normal activity of urothelial cells. Due to special physiological properties of urothelial cells a specific expression and organisation of cytokeratins is required. Why has each layer of urothelial cells its own profile of cytokeratins is not clear yet. Probably have specific combinations of cytokeratins those physical properties that best fit the needs of cells at definite position in tissue. Trajectorial cytokeratin network enables extreme elasticity of differentiated umbrella cells and consequently changing of the cell shape which must adjust to variable volume of urine. This organisation of cytokeratins is perfectly designed for the intensive transport of fusiform vesicles required for accommodation of the surface area of the apical membrane. Changing of the cytokeratin profile in cells during neoplastic transformation gives a strong tool to pathologists and basic scientists to detect tumour cells and to study mechanisms leading to such transformation of a cell program. Further studies of the cytokeratin network in normal and pathologic urothelium will show whether the architecture of the network could become a new differentiation marker to better distinguish between various neoplastic transformations of urothelial cells.

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