Two alternative pathways of asymmetric unit membrane formation in urothelium

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Introduction. The key function of urinary bladder is to store urine and to maintain a tight blood-urine permeability barrier. The barrier depends on the rigid, thickened, and highly insoluble membrane called Asymmetric Unit Membrane (AUM). AUM contains four transmembrane proteins, uroplakins (UPIa, UPIb, UPII, UPIII), which are assembled into 16-nm urothelial particles. Several thousand 16-nm particles together present the primary unit of AUM – an urothelial plaque, which covers the fusiform vesicles and the apical plasma membrane of terminally differentiated superficial urothelial cells. It is suggested that fusiform vesicles in normal urothelium transport AUM into the apical plasma membrane, but the cellular compartments involved in the assembling and in the intracellular trafficking of AUM during formation and maintenance of the urinary bladder permeability barrier are poorly known. Biological (bacterial infections), chemical (toxins in urine) and mechanical (ureterolithiasis) agents can compromise the permeability barrier. Urothelium reacts to the barrier loss with rapid regeneration processes. Hence, the aim of our study was to determine, how AUM is assembled and delivered to the apical plasma membrane of superficial urothelial cells in the normal urothelium and in the urothelium during regeneration. We used cyclophosphamide treatment to remove differentiated urothelial cells, and then morphologically and imunocytochemically followed the de-novo assembly and intracellular trafficking of AUM until complete urothelial regeneration, marked by urothelial differentiation markers (uroplakins, AUM, fusiform vesicles, scalloped luminal surface, cytokeratin 20).

Methods. Bladders of 6-8 week old male mice, strain C57B6 were used. Animals were divided into three groups: (i) untreated, (ii) cyclophosphamide-treated (150 mg/kg bw) and (iii) placebo-treated (0.9% NaCl). On day 0, treated animals received a single intraperitoneal injection of corresponding agents, and were consecutively killed on day 1, 3, 5, and 7 after treatment. For morphological TEM studies, urothelium was rapidly frozen, freeze-substituted, and embedded in Epon. Three dimensional tomography was performed on 300 nm thick sections with a Tecnai 20 (FEI) TEM microscope, running at 200kV. Modeling was done with Imod software (http://bio3d.colorado.edu/imod/). For SEM studies, urothelium was chemically fixed (4% PA+2% GA), critical-point dried, sputtered with gold and examined with a JSM840A (Jeol) SEM microscope, running at 15kV. For immunofluorescence studies, urothelium was chemically fixed (4% PA), washed and labeled with anti-cytokeratin 20 mouse monoclonal antibodies. Pieces of urothelium (3x3 mm) were examined with AxioVizion.Z1 fluorescent microscope with ApoTome upgrade (Zeiss). For immunoelectron microscopy, cryo sections were prepared by modified Tokuyashu method

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and labeled with anti-AUM rabbit polyclonal antibodies, generated against total uroplakins of highly purified bovine AUM (a kind gift of Prof. T.-T. Sun, NYU, USA).

Results. On day 1 after cyclophosphamide treatment, uroplakins and AUM were not detected in the superficial urothelial cells, their cytoplasm contained no fusiform vesicles, luminal surface was covered with microvilli, and cytokeratin 20 was not detected. On days 3-5, increasing amount of uroplakins was detected on the apical plasma membrane. Since day 3, uroplakin-positive rounded vesicles (<100 nm, named UP-transport vesicles) were observed in the cytoplasm, and since day 5, also discoidal vesicles. AUM was first seen on the apical cell surface, but not in UP-transport vesicles. Between days 3-5, the luminal surface covered with microvilli changed, and ropy- and rounded microrigdes appeared. Patches of cytokeratin 20 were detected in the cytoplasm. On day 7 after cyclophosphamide treatment, superficial cells were comparable to the superficial cells of normal urothelium. Uroplakins and AUM were detected on the apical plasma membrane and in the fusiform vesicles. The cytoplasm contained (i) UP-transport vesicles, that were observed close to the *trans*-Golgi network, (ii) discoidal vesicles of various lengths (100-500 nm), either appearing as individual compartments or arranged in stacks, and (iii) fusiform vesicles (600-1200 nm), again either appearing as individual organelles or in stacked organizations in the central part of cytoplasm, and individually in the subapical part of the cytoplasm. Cytokeratin 20 was detected between subapical and central part of the cytoplasm, and was arranged as network. The luminal surface was scalloped-looking.

Discussion. Regeneration of superficial urothelial cells after injury could be divided into three phases: early, central, and final. In the early phase (day 1 after treatment), AUM is not formed. In the central phase (days 3-5 after treatment), uroplakins start to be synthesized in endoplasmic reticulum-Golgi compartments, and are transported as 16-nm uroplakin particles with small UP-transport vesicles directly to the apical cell surface (plasma membrane). AUM is formed at the apical cell surface, which is crucial for rapid establishment of the urinary bladder permeability barrier. In the final phase of regeneration (day 7 post treatment) and in the normal urothelium, the apical cell surface is covered by AUM. In that phase, AUM is formed and stored in the cytoplasm. Its formation is characterized by progressive concentration of 16-nm particles in the post-Golgi compartments: UP-transport vesicles fuse with each other and form discoidal vesicles. In the discoidal vesicles, the sizes of urothelial plaques increase by addition of new uroplakin particles and fusiform vesicles are gradually formed. They are organized in stacks in the central cytoplasm. Individual fusiform vesicles can be transported through the cytokeratin 20 network and subsequently inserted into the apical plasma membrane.

Conclusion. AUM is formed by two alternative pathways. During urothelium regeneration, 16-nm uroplakin particles are transported from the *trans*-Golgi side directly to the apical plasma membrane, and AUM is formed there. In normal urothelium, 16-nm uroplakin particles are first concentrated in the post-Golgi compartments in the cytoplasm, AUM is formed there, and it could later be transported by fusiform vesicles to the apical cell surface.

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