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Endocytotic activity of bladder superficial urothelial cells is inversely related to their differentiation stage

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ABSTRACT

The composition of the apical plasma membrane of bladder superficial urothelial cells is dramatically modified during cell differentiation, which is accompanied by the change in the dynamics of endocytosis. We studied the expression of urothelial differentiation-related proteins uroplakins and consequently the apical plasma membrane molecular composition in relation to the membrane-bound and fluid-phase endocytosis in bladder superficial urothelial cells. By using primary urothelial cultures in the environment without mechanical stimuli, we studied the constitutive endocytosis. Four new findings emerge from our study. First, in highly differentiated superficial urothelial cells with strong uroplakin expression, the endocytosis of fluid-phase endocytotic markers was 43% lower and the endocytosis of membrane-bound markers was 86% lower compared to partially differentiated cells with weak uroplakin expression. Second, superficial urothelial cells have 5–15-times lower endocytotic activity than MDCK cells. Third, in superficial urothelial cells the membrane-bound markers are delivered to lysosomes, while fluid-phase markers are seen only in early endocytotic compartments, suggesting their kiss-and-run recycling. Finally, we provide the first evidence that in highly differentiated cells the uroplakin-positive membrane regions are excluded from internalization, suggesting that uroplakins hinder endocytosis from the apical plasma membrane in superficial urothelial cells and thus maintain optimal permeability barrier function.

1. Introduction

Endocytosis is involved in many important cellular functions including maintaining the composition of the plasma membrane (Polo and Di Fiore, 2006). Therefore, in most cells the endocytotic system is organized following a common concept that allows for the integrative handling of a variety of housekeeping functions (Sachse et al., 2002). In the bladder superficial urothelial cells (SUCs) in which the exchange between toxic urine and cytoplasm must be minimized, the excessive endocytosis might lead to urine internalization.

In the past 20 years only few studies have analysed endocytotic events in bladder urothelium (Lewis and de Moura, 1982; Amano et al., 1988, 1991; Jezernik and Sterle, 1992; Romih and Jezernik, 1994; Zhang and Seguchi, 1994; Truschel et al., 2002; Wang et al., 2005). Studies of fluid-phase endocytosis gave inconsistent results. When horseradish peroxidise (HRP) was injected into the bladder, HRP was observed in some endocytotic compartments after bladder contraction (Amano et al., 1991). But when colloidal gold particles were injected into urinary bladder, no particles were observed in superficial cell cytoplasm, suggesting that no fluid-phase endocytosis occurs in these cells (Amano et al., 1988). On the other hand, the studies of membrane-bound endocytosis revealed that the old apical plasma membrane of SUCs is degraded by lysosomes (Amano et al., 1991; Jezernik and Sterle, 1992; Romih and Jezernik, 1994; Zhang and Seguchi, 1994). Similarly, the membrane exposed to the surface after mechanical stretch (Truschel et al., 2002) or after ATP-induced exocytosis (Wang et al., 2005) is also degraded by lysosomes. However, no study was focused on the possible variations in endocytotic dynamics from the different apical plasma membrane regions of SUCs, i.e. asymmetrical and symmetrical membrane regions. It is known that the apical plasma membrane of terminally differentiated SUCs is almost entirely covered with asymmetric unit membranes (AUMs), consisting mainly of uroplakins (UPs), i.e., UPIa, UPIb, UPII, and UPIIIa (Wu et al., 1990; Kachar et al., 1999; Kong et al., 2004; Hu et al., 2005; Sun, 2006). Between AUMs there are so-called hinge regions of symmetrical membrane (Hicks, 1965). Such a composition of apical plasma membrane of SUCs supports the urothelium function as a blood–urine barrier (Hu et al., 2002).

We have shown previously that during early postnatal development of mice bladder extensive endocytotic activity

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occurs, which is followed by decreased endocytotic activity when SUCs are differentiated (Romih and Jezernik, 1994). Since during differentiation of urothelial cells the uroplakins gradually organize into AUMs (Kreft et al., 2002; Veranič et al., 2004), our hypothesis in the present study was that differentiation stage of SUCs, in particular the molecular composition of apical plasma membrane, regulates the dynamics of membrane-bound and fluid-phase endocytosis in bladder SUCs.

In order to compare the dynamics of endocytosis between partially and highly differentiated SUCs, our previously established primary urothelial culture, which mimics bladder urothelium in vivo, was used (Kreft et al., 2002). There are three main reasons for using this model. First, the urothelial cells in primary explant outgrowths form a multilayered urothelium in which superficial cells are heterogeneous in their differentiation stage as shown by their size and the apical plasma membrane composition (Kreft et al., 2002, 2005). In partially differentiated cells the expression of uroplakins is weak; consequently, the apical plasma membrane of these cells is mainly symmetrical. In contrast, in highly differentiated cells the expression of uroplakins is strong; consequently, AUM regions are predominant over symmetrical membrane regions. Therefore, this model enables the simultaneous comparison of endocytosis between partially and highly differentiated SUCs. Second, the SUCs are polarized and connected with well-developed, claudin-8-positive tight junctions (Kreft et al., 2006); hence, the examination of endocytosis from the apical plasma membrane side, i.e. apical endocytosis, is possible. Third, the mechanical stimuli are not present and therefore the constitutive endocytosis can be evaluated.

In the present study, we have analysed the membrane-bound and the fluid-phase apical endocytosis in the bladder SUCs in vitro. Differentiation of SUCs was determined by immunogold and immunofluorescence labelling of urothelial differentiation-related proteins, uroplakins. The relation between endocytotic activity and differentiation stage of SUCs was quantitatively analysed. In addition, the quantitative analyses were performed to compare the endocytotic activity of bladder SUCs with the endocytotically highly active kidney epithelial cells (MDCK). The lysosomal degradation of endocytotic markers was evaluated by the analysis of colocalization between lysosomal associated membrane protein LAMP-2 and the relevant endocytotic marker.

We provide evidence that the membrane-bound and fluid-phase endocytotic activity from the apical side of the bladder SUCs is dependent on the apical plasma membrane composition of the cells and thus their differentiation stage. In highly differentiated SUCs with strong uroplakin immunolabelling of apical plasma membrane, the endocytosis is inhibited. These data indicate a possible additional physiological role for uroplakins, i.e. hindering the apical endocytosis in the bladder SUCs. The potential clinical significance of the acquired results for targeted drug delivery is discussed.

2. Materials and methods

2.1. Cells and media

The animal experiments were approved by the Veterinary Administration of the Slovenian Ministry for Agriculture and Forestry in compliance with the Animal Health Protection Act and the Instructions for Granting Permits for Animal Experimentation for Scientific Purposes. Urinary bladders were obtained from adult male mice; strain Hsd: ICR (CD-1), (25–30 g). The bladders were handled aseptically and immediately immersed in a medium consisting of equal parts of MCDB 153 medium (Sigma, Taufkirchen, Germany) and Advanced-DMEM (Invitrogen, Gibco, Paisley, UK). Primary urothelial culture was prepared as described previously (Kreft et al., 2002). The endocytosis was studied on 7-day-old explant outgrowths, i.e. primary urothelial cultures.

MDCK cells (a kind gift of Prof. Dr. Boris Turk, Institute of Josef Stefan, Ljubljana, Slovenia) were grown in Advanced-MEM (Invitrogen), supplemented with 4 mM Glutamax (Invitrogen), 2.5% fetal bovine serum (Invitrogen), 100 μg/ml streptomycin and 100 U/ml penicillin. MDCK cells were cultured for 7 days to obtain fully polarized confluent monolayers.

2.2. Materials

The following endocytotic markers were used: lectin wheat germ agglutinin conjugated with FITC (WGA-FITC), wheat germ agglutinin conjugated with colloidal gold 10 nm (WGA-Au), dextran conjugated with FITC (dextran-FITC), and peroxidase from horseradish type II (HRP). All were from Sigma Chemicals, St. Louis, MO. Bovine serum albumin labelled with colloidal gold 10 nm (BSA-Au) was from BBInternational, Cardiff, UK.

The following antibodies (Abs) were used: polyclonal rabbit Ab against uroplakins (anti-AUM) reacts strongly, on immunoblotting, with UPIIIa: moderately with UPIa/Ib and weakly with UPII; monoclonal mouse Ab against UPIIIa (AU1). Both Abs were kind gifts of Prof. Dr. Tung-Tien Sun, Department of Cell Biology, New York University Medical School (Wu et al., 1990); polyclonal rabbit Ab against lysosome associated membrane protein 2 (LAMP-2) was a kind gift of Prof. Dr. Paul Saftig, Unit of Molecular Cell Biology and Transgenic Research, Institute of Biochemistry, Christian Albrecht University Kiel, Germany. Secondary anti-rabbit Abs conjugated with Alexa Flour® 488 or Alexa Fluor® 555 were from Molecular Probes, Leiden, Netherlands; anti-rabbit IgG and anti-mouse IgG, both conjugated to 5 nm colloidal gold, and anti-rabbit IgG conjugated to 10 nm colloidal gold were from Auro Probe TM, Amersham Pharmacia Biotech, UK.

Other reagents used were DAPI-Vectashield (Vector Laboratories, Burlingame, CA, USA), ruthenium red (Standard Fluka, Sigma-Aldrich, Hannover, Germany), IGSS gelatin (Amersham, UK), Lowicryl HM20 (Chemishe Werke Lowi, Waldkraiburg, Germany), and Epon (Serva Electrophoresis, Heidelberg, Germany).

2.3. Endocytosis of membrane-bound endocytotic markers

To determine the binding of WGA on the apical plasma membrane of SUCs, WGA-FITC or WGA-Au was added to the apical side of urothelial cultures grown for 7 days on the porous membrane supports with pore size 0.4 μm (Cell Culture Inserts, BD Falcon, Heidelberg, Germany). During the incubation with lectins at 4 °C, 20 mM Hepes was added in the culture medium to maintain the physiological pH.

To determine the apical endocytosis of membrane-bound endocytotic markers in SUCs, the urothelial cultures were incubated with WGA-FITC (0.2 mg/ml) or WGA-Au (diluted 1:20) at 37 °C for various periods of time (5, 30 min, 1, 2, 5, 12, 24 h) and were then fixed and prepared for fluorescence or electron microscopy, respectively.

For fluorescence microscopy, after incubation in WGA-FITC the urothelial cultures were rinsed with cold PBS (pH 7.2) and fixed with absolute ethanol for 15 min at room temperature in the dark. After fixation, the cultures were washed in PBS, mounted in DAPI-Vectashield for DNA labelling and to prevent bleaching of the fluorochromes, and examined in a fluorescence microscope (Nikon Eclipse TE 300) and in an inverted Zeiss confocal microscope LSM 510. FITC was excited using an argon laser (488 nm).