Rev Physiol Biochem Pharmacol (2015) 168: 1–30
DOI: 10.1007/112_2015_22

Published online: 31 March 2015

Properties of the Urothelium that Establish the Blood–Urine Barrier and Their Implications for Drug Delivery

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Abstract The primary function of the urinary bladder is to store and periodically release urine. How the urothelium prevents permeation of water, ions, solutes, and noxious agents back into the bloodstream and underlying tissues as well as serving as a sensor and transducer of physiological and nociceptive stimuli is still not completely understood, and thus its unique functional complexity remains to be fully elucidated. This article reviews the permeation routes across urothelium as demonstrated in extensive morphological and electrophysiological studies on in vivo and in vitro urothelia. We consider the molecular and morphological structures of urothelium and how they contribute to the impermeability of the blood–urine barrier. Based on the available data, the extremely low permeability properties of urothelium can be postulated. This remarkable impermeability is necessary for the normal functioning of all mammals, but at the same time represents limitations regarding the uptake of drugs. Therefore, the current progress to overcome this most resilient barrier in our body for drug therapy purposes is also summarized in this review.

Keywords Blood–urine barrier · Drug delivery · Paracellular transport · Permeability properties · Transcellular transport · Transepithelial electrical resistance · Uroplakins · Urothelium

Contents

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1 Introduction

The urothelium is a stratified epithelium that lines the urinary tract from the renal pelvis to the proximal urethra, including the urinary bladder (Romih et al. 2005). Since the urinary bladder must be capable of storing urine with potentially noxious levels of urea, ammonia, and other toxic metabolites for prolonged periods of time, the low permeability of the urothelium is essential for the normal functioning of all mammals. Although the urothelium is a multifunctional epithelium also involved in sensory transduction, its maintenance of the blood–urine barrier is its most crucial function. The permeability properties of an epithelium define the movement of molecules and ions across the epithelium, and it is vital that this movement is regulated to ensure homeostasis in any organism. This movement can occur through two distinct mechanisms: paracellular passive diffusion through tight junctions (TJs) and transcellular transport involving endocytosis/exocytosis and transcytosis. Permeability studies of different tissues revealed that urothelium is the least penetrable epithelium found among organisms (Table 1).

2 Urothelial Permeability Is Determined by Its Structural Components

The urothelium is the tightest epithelial barrier in the body (Tables 1 and 2), which would not be possible without the specific molecular and morphological structures of the urothelium. Already the shape of the urinary bladder as a sphere minimizes the ratio of urothelium surface area to urine volume, thus reducing the possibility of passive movement of substances across the bladder wall (Lewis 2000). Furthermore, the urothelium consists of a single layer of small basal urothelial cells (UCs), one to several layers of intermediate UCs, and a layer of highly differentiated superficial UCs, also known as “umbrella cells,” due to their size that enables them to cover underlying cells in a parasol-like fashion (Fig. 1b). UCs are also known to have an extremely slow turnover rate with a cell cycle time of 40 weeks in

Table 1 The permeability of different epithelial tissues to water. Permeability properties between different epithelia and between different species vary greatly, yet the urothelium of the urinary bladder is consistently the least permeable epithelium among most organisms

Epithelial tissue	Species	Permeability coefficient (cm/s)	References
Amniotic membrane	Human	32×10^{-2}	Capurro et al. (1989)
Colon	Human	22×10^{-2}	Capurro et al. (1989)
Cecum	Rat	18×10^{-2}	Capurro et al. (1989)
Gallbladder	Rabbit	15×10^{-2}	van Os et al. (1979)
Brain capillary	Rhesus monkey	1.9×10^{-4}	Eichling et al. (1974)
Cornea	Rabbit	1.5×10^{-4}	Prausnitz and Noonan (1998)
Urinary bladder (urothelium)	Toad	7.9×10^{-2}	Capurro et al. (1989)
	Porcine	8.8×10^{-5}	Sugasi et al. (2000)
	Human	7.7×10^{-5}	Sugasi et al. (2000)
		6.6×10^{-5}	Fellows (1972)
	Guinea pig	5.7×10^{-5}	Lavelle et al. (2000)
	Rabbit	5.1×10^{-5}	Negrete et al. (1996a), Lavelle et al. (2000)
	Rat	4.7×10^{-5}	Lavelle et al. (2000)
	Cat	4.0×10^{-5}	Lavelle et al. (2000)
	Mouse	0.9×10^{-5}	Hu et al. (2002)

mouse (Jost 1989) and a ^{3}H-TdR-labeling index less than 0.1% in rat (Locher and Cooper 1970), which additionally contributes to the impenetrable integrity of the urothelium.

The urothelium has two permeation pathways (Fig. 2). The transcellular pathway consists of the apical and basolateral plasma membranes. The main transcellular permeability barrier is the apical plasma membrane of superficial UCs, which is unique in its many specialized features, such as its superficial glycosaminoglycan layer (Parsons 2007), urothelial plaques composed of transmembrane proteins uroplakins (Hu et al. 2000; Kreft and Robenek 2012), and its particular lipid composition (Grasso and Calderon 2009), which all influence the course of passive diffusion, active transport, and endocytosis. The paracellular pathway consists of intercellular space and TJs, which are extremely impenetrable and represent the main barrier to paracellular transport (Fig. 2).

For nearly all epithelia, the apical plasma membrane and TJs serve as the main barrier to molecular and ionic flux. However, the barrier between urine and blood has been shown to exhibit the highest recorded transepithelial resistance (TER) of all epithelia with a value of up to 78,000 Ωcm^2 (Lewis and Diamond 1976). Nevertheless, other measurements of different bladder urothelia demonstrated lower TER values (Table 2). These differences in resistance values could be due to methodological variations and differences in the bladder stage (distention) or could reflect a genuine difference between species. The values in Table 2 were obtained from excised mucosa from different donor tissues. When working with varying donor

Table 2 The transepithelial resistance (TER) of different epithelia. TER values of different epithelial tissues vary due to their different biological functions, and the blood–urine barrier consistently exhibits the highest TER values among different epithelia of different organisms. Note that all values were obtained from excised mucosa, except cerebral vessels, that were experimented on in situ

Epithelial tissue	Species	TER (Ωcm^2)	References
Gallbladder	Human	66.5	Chinet et al. (1999)
Duodenum	Rabbit	211	Rojanasakul et al. (1992)
Jejunum	Mouse	36	Schulzke et al. (2005)
	Rabbit	224	Rojanasakul et al. (1992)
Ileum	Rabbit	266	Rojanasakul et al. (1992)
Colon	Mouse	64	Schulzke et al. (2005)
	Rat	114	Soler et al. (1999)
	Human	142	Soler et al. (1999)
	Rabbit	288	Rojanasakul et al. (1992)
Nasal cavity	Rabbit	261	Rojanasakul et al. (1992)
Bronchus	Rabbit	266	Rojanasakul et al. (1992)
Trachea	Rabbit	291	Rojanasakul et al. (1992)
Vagina	Rabbit	372	Rojanasakul et al. (1992)
Rectum	Rabbit	406	Rojanasakul et al. (1992)
Amniotic membrane	Mouse	580	(Kobayashi et al. 2010)
Cornea	Rabbit	1,000	Nakamura et al. (2007), Rojanasakul et al. (1992)
Cerebral venous vessels	Rat	918	Butt et al. (1990)
Cerebral arterial vessels	Rat	1,490	Butt et al. (1990)
Buccal cavity	Rabbit	1,803	Rojanasakul et al. (1992)
Urinary bladder (blood–urine barrier)	Rat	1,250	Lavelle et al. (2000)
	Mouse	2,000	Hu et al. (2002)
	Frog	2,000	Martínez-Palomo and Erlij (1975)
	Guinea pig	2,380	Lavelle et al. (2000)
	Rabbit	3,210	Lavelle et al. (2000)
	Cat	3,300	Lavelle et al. (2000)
	Mouse	5,800	Schulzke et al. (2005)

tissues, biological variability in the form of genetic and epigenetic factors must be considered. Additionally, deviations in experimental conditions also result in different electrical properties of cell layers and thus discrepancies in the observed values (Stolwijk et al. 2014). Apart from excised tissues, in vitro cell cultures are becoming ever more widespread for electrical measurements based on Ohm's law to assess epithelial permeability. Filter-based electrical assays often use handheld chopstick electrodes, whereas solid substrate-based impedance methods such as electric cell-substrate impedance sensing can produce reliable and reproducible assays in vitro as well as enable monitoring of wound healing, cell proliferation, and micromotion.

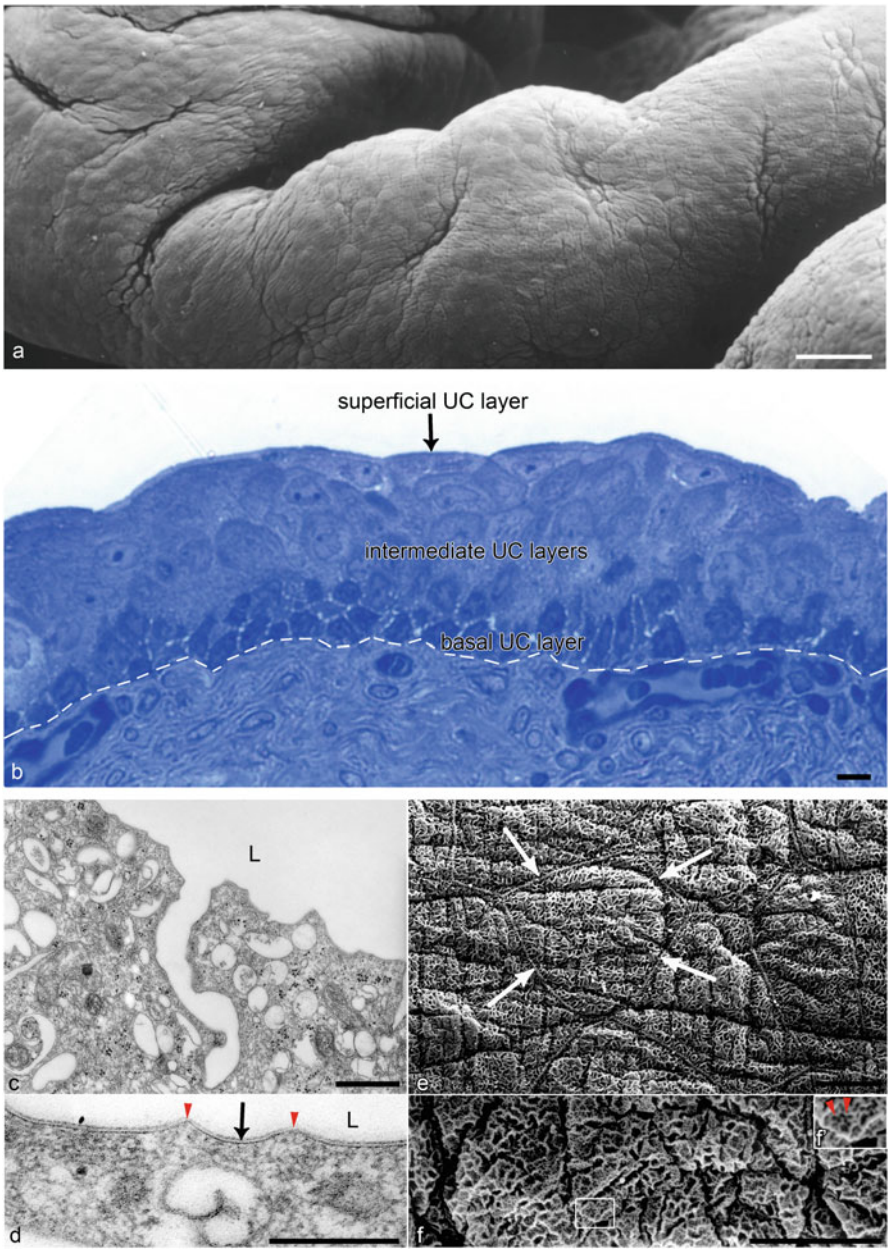

Fig. 1 Porcine urothelium in vivo. (**a**) The surface of the urothelium seen under scanning electron microscopy. (**b**) Epon semithin section of urothelium stained with toluidine blue. The urothelium consists of basal, intermediate, and superficial urothelial cells (UCs). (**c**, **d**) Transmission electron microscopy of apical plasma membrane of superficial UCs. Round- to discoid-shaped vesicles are present in the cytoplasm of superficial UCs, and the apical membrane can be folded. The apical plasma membrane has an asymmetric unit membrane of urothelial plaques (*arrow* in **d**) separated by hinges (*red arrowheads* in **d**) facing the lumen (*L*) of urinary bladder. (**e**, **f**, and **f′**) Scanning electron microscopy of superficial UCs. Note the polygonal shape (*arrows* in **e**) and scalloped

Nevertheless, the reproducibility of in vitro models is also highly susceptible to different sources and isolations, passage number, substrate, seeding densities, and time in culture (Stolwijk et al. 2014). Although experimental techniques may vary, the vast amount of research done on the urothelium demonstrates that the urothelial barrier is undoubtedly an extremely tight barrier that uniquely regulates paracellular and transcellular transport, as is discussed in the following sections.

3 The Paracellular Pathway

Paracellular transport is passive, has no directional discrimination, and consists of diffusion and osmosis (Anderson 2001). The paracellular resistance of the urothelium consists of two resistances in series, the cell–cell junctions and the intercellular cleft (Claude 1978; Powell 1981). The lateral intercellular space is very narrow (0.68 nm) and with its length contributes a distributed resistance in series with the lateral plasma membrane (Clausen et al. 1979). However, the resistance of the intercellular space is usually much less than 10% of the total paracellular resistance (Claude 1978). The amount of current traversing the paracellular pathway depends on the specific resistance of that pathway and on the ratio of the pathway per unit area of epithelial surface. Moreover, the amount of junctions per unit area of epithelium depends on the size and packing of the cells. Superficial UCs are relatively large with a diameter ranging between 25 and 250 μm (Apodaca 2004) and sometimes even larger; it has been shown that within 24 h after injury, some superficial UCs become more flattened and stretched. They measure 150–300 μm in length and are up to three times larger than normal-sized superficial UCs (Kreft et al. 2005). Superficial UCs are also hexagonally packed, thus having less cell–cell contact per unit area (Claude 1978; Anderson and Van Itallie 2009; Jerman et al. 2013). On the other hand, interdigitation of adjacent cells increases the amount of junction per unit area (Claude 1978). Interdigitation is found at the superficial UC borders, and this membrane zipper might contribute to the barrier function. The membrane overlap does not include TJs and is thought to fasten two cells better together by providing more apical membrane surface contact, additionally increasing the electrical resistance of the junction and possibly stabilizing the TJs (Kreplak et al. 2007; Višnjar et al. 2012; Višnjar and Kreft 2013).

TER is a measure of ion permeability (Lewis 2000), and the main route of passive ion permeation does not traverse the cells, but the cell junctions. According to the magnitude of TER and the relative resistance of transcellular to paracellular

Fig. 1 (continued) appearance of the apical surface of the superficial UCs (**f**). The hinges in (**d**) correspond to the microridges seen in (**f**) and (**f´**) (*red arrowheads* in enlarged *inset*). Inside the microridges lies the urothelial plaque, as we demonstrated in Zupančič et al. (2014b). *Large inset* framed with *white lines* is twice enlarged image of the corresponding small *white-framed inset*. Bars: 100 μm (**a**), 10 μm (**b**, **e**, **f**), 500 nm (**c**), 200 nm (**d**)

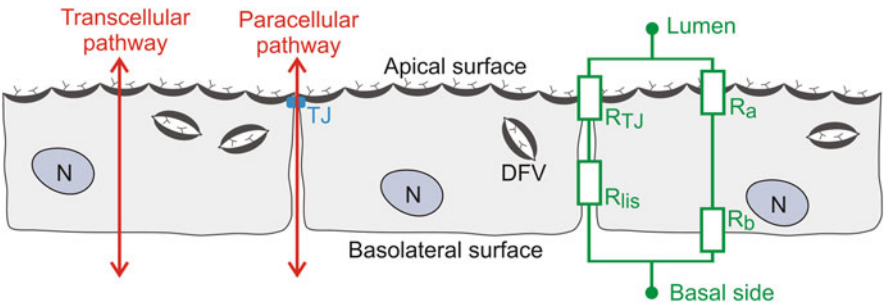

Fig. 2 Paracellular and transcellular pathways of the urothelium. The paracellular pathway represents the intercellular route between the urothelial cells (UCs). The gatekeepers of the paracellular pathway are the tight junctions (TJs) at the apicolateral junctions of superficial UCs. The transcellular pathway represents the route across the cell via plasma membrane channels, carriers, exchangers, endo-/exocytosis, and diffusion. The apical plasma membrane is represented with urothelial plaques and glycocalyx. The critical components that contribute to the resistance of the urothelium: R_{TJ} tight junction resistance, R_{lis} lateral intercellular space resistance, R_a apical membrane resistance, and R_b basolateral membrane resistance. *DFV* discoidal fusiform vesicles, *N* nucleus, *TJ* tight junction

pathways, epithelia are divided into "leaky" epithelia, with lower TER values (6–133 Ωcm^2), or "tight" epithelia with higher TER values (365–2,000 Ωcm^2) (Fromter and Diamond 1972). An additional study further classified epithelia into "intermediate to tight" (300–600 Ωcm^2) or "very tight" epithelia (1,000–2,000 Ωcm^2) (Claude and Goodenough 1973). According to these criteria, the urothelium is a tight epithelium (Table 2). Tight epithelia display reduced passive leakage of ions and can thus maintain steeper electrochemical gradients produced by active transcellular transport (Fromter and Diamond 1972). Epithelia with high resistances were observed to have TJs with more strands than low-resistance epithelia. Thus, it was hypothesized that there is an exponential relationship between the number of TJ strands and passive resistance (Claude 1978; Furuse et al. 2001). Now we know that it is indeed the structure of TJs, specifically their variable constitutions of claudins, that is responsible for the magnitude of transepithelial conductance.

3.1 Tight Junctions

The intercellular junction complexes between cells play an important role in epithelial adhesion and barrier function and consist of adherens junctions, gap junctions, desmosomes, and TJs. TJs are the most apical junction complexes at the border between apical and basolateral membrane and are the crucial mediators of paracellular transport (Balkovetz 2006). The TJ has a gate function, which restricts paracellular diffusion between adjacent cells, and a fence function, which maintains polarity of the apical and basolateral exoplasmic leaflets (Acharya

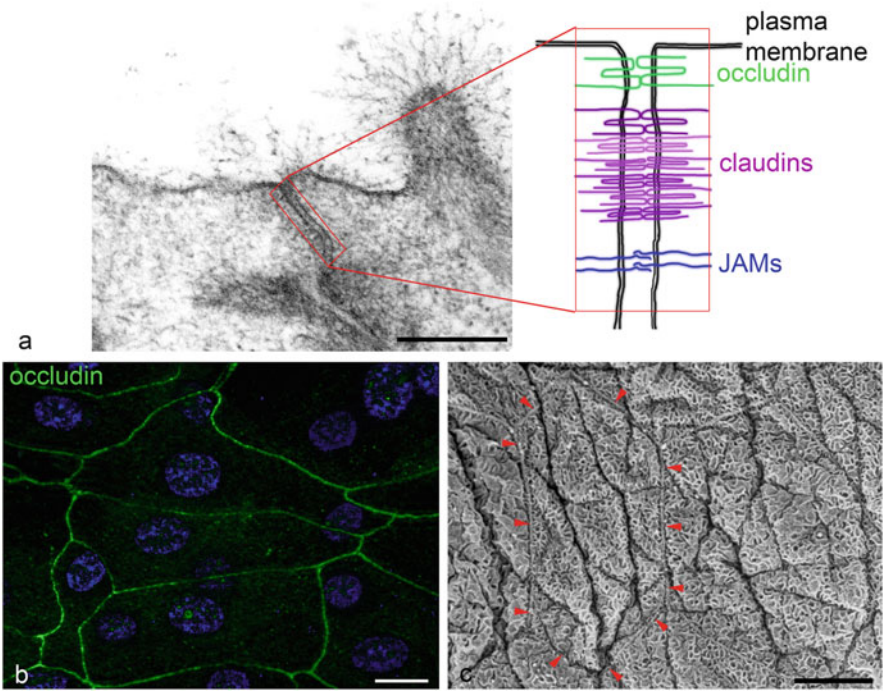

Fig. 3 Urothelial tight junctions (TJs). TJs between the urothelial cells (UCs) are seen as a branching network of transmembrane protein strands that encircle UCs apically and connect two superficial UCs. (**a**) There are three members of transmembrane proteins: junctional adhesion molecules (JAMs), claudins (CLs), and occludin. (**b**) Occludin (*green*) is one of the first expressed TJ proteins that can be seen in UC cultures at the junction between two adjacent cells. UC cultures were prepared as described in Višnjar and Kreft (2015). Nuclei are *blue*. (**c**) When all the TJ proteins are expressed and located in the right position, the formation of TJs is complete (*red arrowheads*). Bars: 200 nm (**a**), 10 μm (**b**, **c**)

et al. 2004). The TJs are close cell–cell contacts with continuous rows of transmembrane proteins, which determine the permeability of the paracellular barrier by providing a semipermeable size- and ion-specific barrier. The TJ consists of peripherally associated scaffolding proteins, cytosolic signaling proteins, and transmembrane proteins. *Zonula occludens*-1 (ZO-1) is a scaffolding protein, which organizes transmembrane proteins and binds to cytoplasmic proteins and actin filaments (Anderson 2001). The transmembrane proteins of the TJ include junctional adhesion molecules (JAMs), occludin, and claudins (CLs) (Fig. 3).

Occludin spans the membrane four times, undergoes homophilic adhesion between cells (Anderson 2001), and most likely stabilizes the TJ, enhancing barrier function (McCarthy et al. 1996; Lacaz-Vieira et al. 1999; Al-Sadi et al. 2011; Cummins 2012). All CLs consist of four transmembrane domains, two extracellular loops, and a short COOH intracellular tail (Tsukita et al. 2001). The amino acid composition of the extracellular loop determines TJ paracellular conductance by determining the characteristics of the pore, basically serving as an "electrostatic

Table 3 Expression of claudins (CLs) in urothelial cells (UCs). CLs are differentially expressed, depending on cell localization and differentiation. However, certain studies have demonstrated variable expression of the same CLs (i.e., CL1, CL4, CL7, CL8, and CL12). TEU-2 cells, immortalized human ureteral cell line; RT112/84, human bladder carcinoma epithelial cell line

Claudin	Localization of expression	Species/cells	References
CL1	Proliferating UCs	Human cells	Varley et al. (2006)
	Plasma membrane of basal and intermediate UCs	Human	Nakanishi et al. (2008)
			Székely et al. (2011)
			Törzsök et al. (2011)
	Plasma membrane at borders between superficial UCs	TEU-2 cells	Rickard et al. (2008)
		Human cells	Cross et al. (2005)
CL2	Cytoplasm of UCs	TEU-2 cells	Rickard et al. (2008)
	mRNA, under the apical membrane of UCs in all three layers	Mouse and rat	Acharya et al. (2004)
CL3	Plasma membrane at borders between superficial UCs	Human cells	Varley et al. (2006)
			Varley and Southgate (2008)
	Apicolateral surface of superficial UCs	Human	Nakanishi et al. (2008)
			Törzsök et al. (2011)
			Smith et al. (2015)
CL4	Plasma membrane at borders between superficial UCs	Human cells	Varley et al. (2006)
			Varley and Southgate (2008)
			Cross et al. (2005)
		TEU-2 cells	Rickard et al. (2008)
	Plasma membrane at borders between UCs in all three layers	Mouse, rabbit, and rat	Acharya et al. (2004)
			Fujita et al. (2012)
		Mouse cells	Kreft et al. (2006)
	Basolateral surface of superficial UCs	Human	Nakanishi et al. (2008)
			Székely et al. (2011)
			Törzsök et al. (2011)

(continued)

Table 3 (continued)

Claudin	Localization of expression	Species/cells	References
CL5	Plasma membrane at borders between superficial UCs	Human cells	Varley et al. (2006)
		TEU-2 cells	Rickard et al. (2008)
		Human	Törzsök et al. (2011)
CL7	Plasma membrane of all three UC layers	Human	Nakanishi et al. (2008)
			Törzsök et al. (2011)
	Plasma membrane at borders between intermediate and basal UCs	Human cells	Varley et al. (2006)
CL8	Cytoplasm of UCs	TEU-2 cells	Rickard et al. (2008)
	Plasma membrane at borders between superficial UCs	Mouse and rat	Acharya et al. (2004)
		Mouse cells	Kreft et al. (2006)
		Porcine cells	Višnjar et al. (2012)
			Višnjar and Kreft (2013)
			Visnjar and Kreft (2014)
CL11	Plasma membrane at borders between UCs	RT112/84	Awsare et al. (2011)
CL12	Cytoplasm of UCs	TEU-2 cells	Rickard et al. (2008)
	Plasma membrane at borders between superficial UCs	Mouse and rat	Acharya et al. (2004)
CL13	Only mRNA detected in bladder tissue	Mouse	Acharya et al. (2004)
CL14	Borders of superficial UCs	TEU-2 cells	Rickard et al. (2008)
CL16	Borders of superficial UCs	TEU-2 cells	Rickard et al. (2008)

selectivity filter" (Van Itallie and Anderson 2004; Anderson and Van Itallie 2009). CLs are developmentally regulated and expressed in tissue- and cell-specific manners that directly determine paracellular permeability. There are at least 27 CL isoforms (Tsukita and Furuse 1999; Mineta et al. 2011), which are capable of heterophilic and homophilic adhesion between cells and are responsible for the variable properties of the barrier (Anderson 2001; Angelow et al. 2008). CL expression is variable and in some cases controversial, since some family members exhibit differentiation- and cancer-specific expression. The composition of TJs in

urothelia displays interspecies differences and could be one of the reasons for TER and permeability variance between different species. Additionally, the role, organization, and regulation of the non-tight junction pool of CLs that we have already shown (Kreft et al. 2006) in the basolateral plasma membrane of superficial, intermediate, and basal UCs are still unknown. So far, the research has demonstrated that different CLs are expressed in differently differentiated UCs, indicating that the constitution of CLs in TJs changes with the differentiation stage of UCs and permeability level of urothelium (Table 3). Certain observations on the localization of given CLs, such as CL1, CL2, CL4, CL7, CL8, and CL12, are still inconsistent (see Table 3), and further research needs to be done concerning the expression and localization of a number of CLs in the urothelium.

ZO-1 and occludin are localized to the TJs and basolateral surface of the superficial UCs and the plasma membranes of intermediate and basal cells (Acharya et al. 2004; Kreft et al. 2006). The presence of the TJ proteins ZO-1, occludin, and CL4 at cell borders could help cell adhesion, which would be necessary in tissues undergoing wound healing or mechanical stress, as does the bladder wall (Acharya et al. 2004; Kreft et al. 2006). CL4 also decreases paracellular conductance by selectively decreasing sodium permeability (Van Itallie et al. 2001). CL2 is associated with leaky epithelia (Kiuchi-Saishin et al. 2002), because it increases cation permeability (Amasheh et al. 2002), and was not found in superficial UC TJs (Acharya et al. 2004). On the other hand, CL8 downregulates CL2 expression and decreases cation permeability (Yu et al. 2003). The paracellular barrier seems to depend most on the claudin-based pores that have a radius of 0.4 nm and regulate TJ permselectivity (the ability to discriminate according to ionic charge (Powell 1981)). For example, the ionic charge selectivity of TJs has a preference for Na^+ over Cl^- (Anderson and Van Itallie 2009). There might be a regulation of TJ permeability properties via rapid changes in the composition or half-life of CLs within the TJs. The large variability of TJ paracellular permeability is due to the large number of possible combinations of numerous different CL isoforms, which determine the epithelial TJ gate function (Balkovetz 2006). The two pathway model proposes that molecules smaller than 0.4 nm are discriminated by CL pores according to their size and charge, while molecules larger than 0.4 nm can only traverse through temporary discontinuities in the TJ contacts with no charge selectivity. Although the CL pore can act as a filter, it is wider than transmembrane pores and is hence less discriminatory for ions (Anderson and Van Itallie 2009).

4 The Transcellular Pathway

Alongside the paracellular pathway runs the transcellular pathway with two parallel resistances in series, the apical and basolateral plasma membranes of UCs (Claude 1978; Powell 1981). The intracellular cytoplasm is also part of the transcellular pathway; however, its resistance is negligible. The apical plasma membrane

displays a resistance of up to 150,000 Ωcm^2 (Lewis et al. 1976), which is much higher than that of the basolateral plasma membrane, which has a resistance of only 1,500 Ωcm^2 (Clausen et al. 1979). Transcellular transport is directional and energy dependent, can be passive or active, and is facilitated by transporters and channels (Anderson 2001). Along with electrodiffusion via conductive channels also occurs electroneutral and electrogenic primary and secondary active transport (Frings et al. 1990).

4.1 Plasma Membrane Transport in UCs

4.1.1 Ion Transport and Sensory Transduction

The apical plasma membrane of urothelium contains sodium, potassium, and calcium channels, as well as other cation-sensitive channels. The basolateral plasma membrane of urothelium also contains sodium and potassium channels as well as chloride channels, Na^+/H^+ and Cl^-/HCO_3 exchangers, and ATPase pumps. The urothelium expresses a hormonally regulated active transport system for Na^+ absorption (Lewis 2000), and the bladder actively reabsorbs Na^+ to prevent its loss from the body (Lewis and Diamond 1976). The sodium-dependent cellular pathway is located in the apical plasma membrane of UCs (Cross et al. 2005) and helps move Na^+ from urine to blood down a net electrochemical gradient. This amiloride-sensitive pathway is also permeable to K^+, H^+, and Li^+ because the amiloride-sensitive channels degrade with time (Lewis and Wills 1983; Frings et al. 1990), due to trypsin-like serine proteases in urine such as urokinase, plasmin, and kallikrein (Lewis and Clausen 1991). Cytoplasmic intracellular vesicles of UCs are also observed to contain epithelial sodium channels (Lewis and Clausen 1991; Smith et al. 1998), and stretching causes fusion of these vesicles to the apical plasma membrane, adding epithelial sodium channels and thus increasing Na^+ selectivity (Frings et al. 1990). The apical plasma membrane of superficial UCs also expresses an amiloride-insensitive, oxytocin-stimulated cation channel for Ca^{2+}, as well as a voltage-sensitive channel for K^+. The renal outer medullary potassium channels are localized to the apical plasma membrane of superficial UCs and could regulate the transmembrane electrical potential, sensory transduction, composition of extracellular and intracellular K^+ concentrations, and thus cell volume and osmolality (Spector et al. 2008).

Na^+ conductance is also found in the basolateral membrane of superficial UCs that expresses a barium-sensitive, voltage-sensitive sodium channel as well as a lidocaine-sensitive potassium channel (Frings et al. 1990). Na^+ is actively exported across the basolateral membrane through a Na^+–K^+-activated, ATPase pump, resulting in a net Na^+ current traversing the superficial UCs. Uptake of K^+ into the cell occurs via the Na–K ATPase (Smith et al. 1998; Cross et al. 2005), which enables K^+ to diffuse back into the blood and maintains an electrochemical gradient (Lewis et al. 1978). Additionally, ion transport is affected by mechanical stimuli

(Araki et al. 2008; Yu et al. 2009), and it has been shown that increased hydrostatic pressure increases Na^+ absorption as well as K^+ and Cl^- secretion (Wang et al. 2003b).

Along with mechanosensitive ion channels, the urothelium contains many other receptors, which contribute to sensory transduction of the bladder wall, and it is becoming more and more evident that the urothelium is also a sensory web, playing a role in transmitting information, with numerous sensory afferent and efferent nerve processes. Much is being discovered concerning the sensory properties of urothelium, and for a thorough review of urothelial signaling, see Birder (2010). A considerable amount of attention is currently being focused on the transient receptor potential (TRP) cation channels, which may play a role in the mechano- and/or chemosensory function of bladder urothelium, and functional expression of TRPV4, TRPV2, and TRPM7 (Everaerts et al. 2010a) as well as TRPC1 and TRPC4 (Boudes et al. 2013) has been demonstrated in urothelium. More specifically, TRPV4 seems to play a role in the transduction of intravesical mechanical pressure and thus bladder function (Gevaert et al. 2007). This channel as well as other TRP channels is gaining attention as possible new drug targets for treatment of different urinary tract and bladder pathophysiologies (for more, see Sect. 5). Apart from the TRP channels, the urothelium receives many inputs through a vast number of other surface receptors and ion channels such as nicotinic and muscarinic receptors, the purinergic P2X family of ATP receptors, TRAAK and TREK-1 channels, and acid-sensitive ion channels (Birder 2005; Araki et al. 2008; Khandelwal et al. 2009; Sterle et al. 2014). These surface receptors and ion channels may alter membrane conductance and turnover of the urothelium and should also be considered in the context of urothelial permeability and as possible drug targets.

4.1.2 Urea Transport

The uniquely specialized apical plasma membrane of superficial UCs is the major barrier to permeation. The apical plasma membrane represents 80%, 96%, 97%, and >99% of the resistance to urea, water, ammonia, and proton flux, respectively (Negrete et al. 1996a). Unlike natural physiological concentrations of substances in urine, nonphysiological concentrations can affect the barrier function of the urothelium. It has been demonstrated that long-term exposure to serosal urea irreversibly increases the transepithelial conductance at the TJ, whereas short-term exposure to serosal urea increases ion permeability at the apical plasma membrane (Lewis and Kleine 2000).

Although possible simple diffusion of urea could be considered, the degree to which urea concentrations in urine can change implies facilitated or active transport. Urea and creatinine are constantly reabsorbed from urine (Spector et al. 2007), and the net transport of urea and creatinine is regulated by hydration status. However, there is still controversy on how dehydration affects urea transporter B (UT-B) expression and urea reabsorption (Spector et al. 2004, 2011; Lucien

et al. 2005). UT-B is strongly localized in all urothelial plasma membranes, except the apical plasma membrane of superficial UCs, implying its role in dissipating urea that apically enters the superficial UCs through leakage or diffusion. UT-B might regulate cell volume, osmolality, and net transport of urea across urothelia (Spector et al. 2004; Lucien et al. 2005). Alongside UT-B, urea can be transported via other urea transporters and even certain aquaporins (e.g., AQP-3), reviewed in Sands et al. (1997) and Fu and Lu (2007).

4.1.3 Water Transport

Aquaporins (AQPs) are transmembrane proteins that mainly transport water, but are also capable of transporting other uncharged molecules such as urea, glycerol, and pyrimidines (Fu and Lu 2007). Numerous studies have demonstrated net water influx, and several AQPs have been localized to bladder tissue (Fig. 4). AQP-2 and AQP-3 are localized to all plasma membranes except the apical plasma membrane of superficial UCs, and dehydration causes an upregulation of their expression (Spector et al. 2002). AQP-3, AQP-4, and AQP-7 are expressed in the cytoplasm of proliferating cell cultures and are relocalized to the plasma membrane in differentiated cultures. AQP-9 is only expressed in differentiated cultures, its expression possibly indicating terminal differentiation (Rubenwolf et al. 2009). AQP-1, AQP-2, and AQP-4 are orthodox water-transporting aquaporins, whereas aquaglyceroporins AQP-3, AQP-7, and AQP-9 can transport other uncharged molecules (Fu and Lu 2007). It is now presumed that AQPs can modify urine composition. It has been shown in vivo that inhibiting AQPs with $HgCl_2$ decreases water and urea flux and that hyperosmotic conditions with NaCl increase AQP-3 expression tenfold, whereas hypoosmotic conditions decrease AQP-3 expression by 30%. Urine osmolality ranges from 50 to 1,300 mosm/kg, and higher osmotic gradients increased water and urea permeability coefficients of urothelial in vivo constructs as a result of higher AQP activity (Rubenwolf et al. 2012). In the urothelium, AQPs could form a pathway for water, regulating transepithelial bulk water movement along with cell tonicity and volume (Spector et al. 2002).

4.1.4 Carbon Dioxide Transport

Even though plasma membranes are freely permeable to CO_2, the urothelium is extremely impermeable to CO_2, and partial CO_2 pressure in urine is two- to fourfold higher than in blood, even reaching values from 80 to 100 mm Hg in acidified urine (DuBose 1982; Zocher et al. 2012). CO_2 permeability is not limited by a physical barrier nor affected by the presence of AQPs or uroplakins, but instead by CO_2 hydration/dehydration kinetics – a lack of carbonic anhydrase in the urothelium causes the low CO_2 permeability in the bladder (Zocher et al. 2012). Nevertheless, certain physical barriers play a crucial role in the low permeability properties of the urothelium.

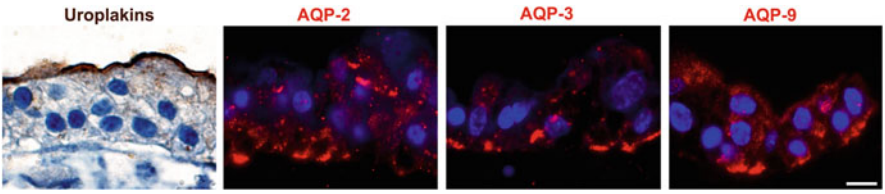

Fig. 4 Uroplakin and aquaporin (AQP) expression in urothelial constructs on stromal amniotic membrane scaffolds in vitro prepared as described in Jerman et al. (2013). Note uroplakins at the apical plasma membrane of superficial urothelial cells (UCs) in urothelial constructs, suggestive of terminal differentiation. All three AQPs are most strongly expressed at the basal membrane of UCs and never at the apical membrane of superficial UCs. Expression of certain AQPs and their relocalization to the plasma membrane may be indicative of higher differentiation stages. Paraffin sections were immunolabeled with antibodies against uroplakins (anti-AUM), anti-AQP-2 (*C-17*, *Santa Cruz*), anti-AQP-3 (*C-18*, *Santa Cruz*), and anti-AQP-9 (*H-40*, *Santa Cruz*). Nuclei are *blue*. Bar: 10 μm (for all images)

4.2 The Mucin Layer

One of the key urothelial permeability physical barriers is the glycosaminoglycan (GAG) layer covering the lumenal plasma membrane of superficial UCs (Fig. 5). The GAG layer acts as a nonspecific antiadherent barrier with antibacterial activity and regulates transepithelial molecular movement (Lilly and Parsons 1990; Hauser et al. 2009). It has been shown that urinary GAG also originates in the kidney and is composed of heparan sulfate, dermatan sulfate, chondroitin sulfate, and hyaluronate (Hurst et al. 1987). Recently, it has been suggested that chondroitin sulfate is the main urothelial luminal GAG that contributes to urothelial barrier function (Janssen et al. 2013). The sulfated polysaccharides of GAGs are negatively charged polyanionic molecules, rendering them hydrophilic, and thus have a high affinity for preferentially binding water ionically (Lilly and Parsons 1990; Parsons et al. 1990). Protamine sulfate is an inactivator of the sulfated polysaccharides, thus reducing water content in the urothelium, and it has been shown that protamine treatment of bladder tissue causes increased absorption of urea, water, and Ca^{2+} and also decreases TER (Parsons et al. 1990; Lavelle et al. 2002). Furthermore, UCs displayed a decrease in GAGs in cases of interstitial cystitis, where increased permeability was found (Hurst et al. 1996). Apart from the GAG layer, the apical plasma membrane of superficial UCs also serves as a physical barrier due to its unique transmembrane proteins uroplakins (UPs).

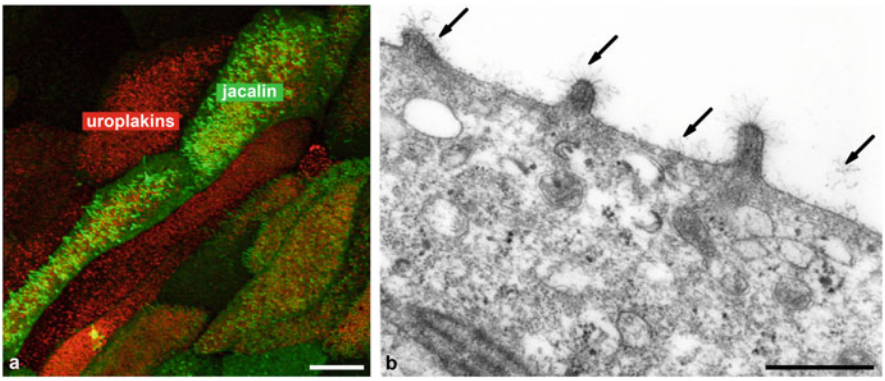

Fig. 5 Glycocalyx of porcine urothelial cells (UCs) in vitro. (**a**) UCs in vitro, prepared as described in Višnjar and Kreft (2015), labeled with the lectin jacalin (*green*) and uroplakins (*red*, antibodies against uroplakins (anti-AUM)). Jacalin binds to *O*-glycosidically linked oligosaccharides, preferring the structure galactosyl (β-1,3) *N*-acetylgalactosamine. (**b**) Glycosaminoglycans of UCs in vitro (*arrows*) seen by transmission electron microscopy. Bars: 10 μm (**a**), 500 nm (**b**)

4.3 Uroplakins

The apical plasma membrane of the superficial UCs is full of plaques, which cover 70−90% of the luminal cell surface (Staehelin et al. 1972) (Fig. 6). The apical cell surface has a scalloped appearance due to the protruding hinge regions of unthickened membrane between plaques, which are concave (Fig. 1d) due to the density difference between the two bilayer leaflets (Kreplak et al. 2007). These plaques are made up of 2D crystals consisting of four major transmembrane proteins uroplakins (UPs). UPIa and UPIb are tetraspanins, while UPII and UPIIIa are single-span proteins. Moreover, latest sequencing of genomes has shown the existence of three new UPII/UPIII family members: UPIIb, UPIIIc, and UPIIId (Desalle et al. 2014). Urothelial plaques seem to reduce membrane permeability on more levels. The UP particles are arranged into a crystalline lattice and contain a large central 6 nm hole, indicating that the majority of the plaque surface (~62%) contains lipids (Min et al. 2003). The crystalline web of rings could organize and restrict lipid movement, lessening membrane fluidity. It has been hypothesized that a rigidified membrane not only decreases permeability but hinders endocytosis, preventing internalization of extracellular substances (Kreft et al. 2009b). On the contrary, a new study suggests that the apical membrane of superficial UCs is hypercompliant, which further demonstrates the complexity of this plasma membrane (Mathai et al. 2014). Furthermore, the different UP subdomains that are embedded in the cytoplasmic and exoplasmic leaflets cause the extracellular domains to be larger than the cytoplasmic domains, creating asymmetry and also suggesting that the leaflets have different lipid compositions. Each single bilayer leaflet can act as independent barrier to permeability, thus contributing to the barrier

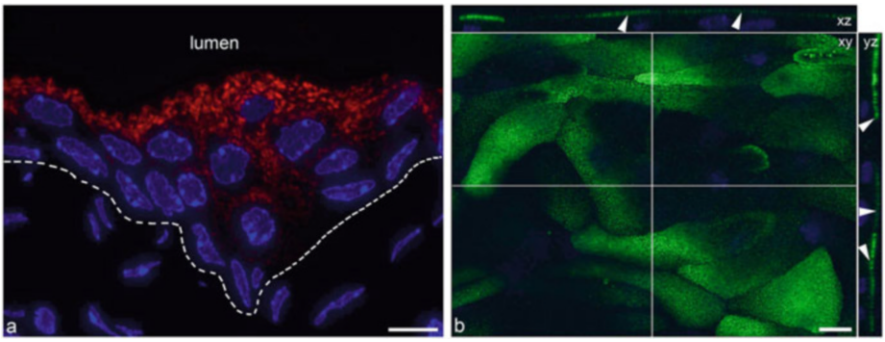

Fig. 6 Uroplakins (UPs) in urothelium of mouse bladder in vivo and in porcine urothelial cells (UCs) in vitro. (**a**) Expression and location of UPs (antibodies against uroplakins (anti-AUM), *red*) in mouse urinary bladder. UPs are seen in superficial and intermediate cells. Nuclei are *blue*. (**b**) Expression and localization of UPs (anti-AUM, *green*) in porcine *UCs* in vitro cultured as is described in Višnjar and Kreft (2015). UPs (*white arrowheads*) are seen above the nuclei (*blue*). Bars: 10 μm (**a**, **b**)

function of the urothelium (Negrete et al. 1996b; Hill and Zeidel 2000; Min et al. 2003). The active role of UPs in the permeability barrier function was demonstrated with UP ablation. Knockout animals with an UPIIIa-deficient urothelium had few plaques, smaller superficial UCs with microvilli, and small round immature discoidal vesicles. Although the UPIIIa-deficient urothelium showed normal TER values, indicating that UPs do not affect TJs, the knockout animals displayed increased water and urea permeabilities (Hu et al. 2002).

4.4 Membrane Lipid Composition

Membrane lipid composition is an important factor in the barrier function of UCs, and lipids of UCs are rich in sphingolipids (mainly cerebroside) and cholesterol, which play an essential role in this barrier (Hicks et al. 1974; Vergara et al. 1974; Stubbs et al. 1979; Hill and Zeidel 2000). Altered lipid compositions influence UP interactions, modifying and possibly even diminishing the amount of UPs (Bongiovanni et al. 2005). Furthermore, endocytosis, vesicle recycling, and lysosomal degradation are also influenced by lipid membrane composition. Compared to controls, oleic acid-derived superficial UCs have been shown to display decreased endocytosis of fluid and membrane-bound probes by 32% and 49%, respectively (Grasso and Calderon 2013). In addition to altered intracellular vesicle trafficking, endocytosed vesicles are capable of releasing their content, and the lipid membrane composition can affect the degree of leakage into the cytoplasm (Grasso and Calderon 2009). Superficial UC plasma membranes, with their urothelial plaques and rigidified lipids, should maximally prevent leakage of toxic urine substances into their cytoplasm, and apical endosomes do indeed exhibit very low

permeabilities to water, urea, and NH_3 (Chang et al. 1994). In fact, apart from TJs and the molecular structure of the apical plasma membrane, the innate cell process of endocytosis is also vital in maintaining the barrier function of the urothelium.

4.5 Exocytosis and Compensatory Endocytosis

The bladder undergoes cyclical changes in volume, which must be accommodated for with structural and cellular changes. Superficial UCs adjust to the increase in urine volume by undergoing morphological changes and inducing vesicle exocytosis (Wang et al. 2003a; Apodaca 2004). Unstretched superficial UCs are cubical with their cytoplasm extended downwards, contain numerous DFVs (Fig. 1c), and have folded basolateral and apical plasma membranes (Fig. 1c). On the other hand, stretched superficial UCs are longer with decreased depth, contain few DFVs, and have flattened apical and basolateral membranes (Truschel et al. 2002).

During stretch, the rate of exocytosis must overtake the rate of endocytosis (Truschel et al. 2002). These opposing processes are most likely coupled to precisely attune the surface changes and replace old damaged plaques and Na^+ channels (Chang et al. 1994; Truschel et al. 2002). Stretch-induced exocytosis is followed by compensatory stretch-induced endocytosis of the apical membrane (Truschel et al. 2002; Khandelwal et al. 2010), which leads to the delivery of endocytosed membrane to the lysosome and degradation by a leupeptin-sensitive pathway (Truschel et al. 2002). Exocytosis in superficial UCs occurs via fusion of subapical DFVs, whereas endocytosis bears peripheral junction-associated apical endosomes (Khandelwal et al. 2010). Most DFVs are probably synthesized de novo (Apodaca 2004; Khandelwal et al. 2008). Voiding results in a third of the apical membrane being endocytosed, confirming a nearly complete recovery of membrane. Endocytotic activity of superficial UCs is probably dependent on the GTPase dynamin-2 (Terada et al. 2009) together with the β_1-integrin-associated signaling pathway, actin, and RhoA and is independent of caveolins, clathrin, and flotillin (Khandelwal et al. 2010). The compensatory endocytosis pathway terminates in lysosomal degradation, and there is little evidence of recycling (Khandelwal et al. 2010). Superficial UCs exhibit the ability for exocytosis and endocytosis as a result of mechanical stimuli; however at rest, constitutive endocytosis seems to be greatly diminished.

4.6 Constitutive Endocytosis

Superficial UCs display elevated endocytotic activity that regulates their differentiation during early postnatal development, but their endocytotic activity decreases significantly as they mature (Romih and Jezernik 1994). Minimal constitutive endocytosis contributes to the permeability barrier by minimizing internalization

of potentially toxic substances. The downregulation of endocytosis could be due to the inability of the large rigid UP plaques to be internalized or the rearranged cytoskeleton (with diminished actin filaments, disassembled microtubules) (Kreft et al. 2009a). Highly differentiated superficial UCs exhibit 43% and 86% less endocytosis of fluid-phase and membrane-bound markers, respectively, than partially differentiated superficial UCs and also display 5–15 times lower endocytotic activity than MDCK cells (Kreft et al. 2009b). However, due to the large variations between the amount of compensatory and constitutive endocytosis, further research should be done concerning the mechanisms regarding both types of endocytosis as well as their activity during voiding versus resting conditions.

Understanding the mechanisms taking place in the bladder in vivo and the molecular and morphological properties of the urothelium are crucial in finding ways to overcome this natural barrier for therapeutic means. With better knowledge of structures such as UPs, TJs, and GAGs, permeation of the urothelium can be used for intravesical drug delivery.

5 The Urothelium and Targeted Therapeutics

The urothelium is implicated in a wide variety of bladder disorders ranging from interstitial cystitis and bladder cancer to numerous other urinary tract and bladder dysfunctions. There has been much focus on urothelial bladder cancer, which is 6th most prevalent among all cancers worldwide (Van Batavia et al. 2014) and causing an estimated 150,000 deaths per year (Jemal et al. 2011). There are two distinct clinical phenotypes of bladder cancer: (a) superficial or non-muscle-invasive bladder cancer and (b) muscle-invasive bladder cancer. In the past decade, numerous bladder cancer biomarkers have been identified (for more, see reviews from Cheng et al. (2014) and Ye et al. (2014)) that in the future could help evaluate disease aggressiveness, progression risk, and overall probability of recurrence. However, the current standard treatments for non-muscle-invasive bladder cancer include transurethral resection of bladder tumors, immunotherapy, or chemotherapy. Muscle-invasive bladder cancer treatment includes surgery and radiation combined with chemotherapy. Unfortunately, there are no alternative treatments, when the initial therapy does not work and no approved targeted agents for this type of bladder cancer exist. Until now, different targeted therapies have been developed and tested or are in ongoing urothelial carcinoma clinical trials, reviewed in Richter and Sridhar (2012) and Ghosh et al. (2014). Besides urinary bladder cancer, one of the prevailing bladder pathophysiologies is interstitial cystitis, which can lead to bladder dysfunction and pain. TRP channels are believed to play a key role in bladder sensation, and TRPV4 is considered a possible target for treating bladder dysfunction through its inhibition and thus reduced transduction of intravesical pressure (Everaerts et al. 2010b). Additionally, studies indicate that TRPC1 and TRPC4 are involved in hyperinnervation of the bladder in cases of bladder cystitis that results in overactive bladder disease (Boudes et al. 2013). For a comprehensive review of the critical role of TRP channels in lower urinary tract dysfunction, see Franken et al. (2014).

5.1 *Enhancing Urothelial Permeability for Intravesical Drug Delivery*

It is crucial that when new therapeutic agents are designed, the extreme impermeability of the urothelium is taken into account, as it represents the primary obstacle to administering drugs. Intravesical drug delivery is the most widely used treatment for administering drugs to the urothelium, because of the advantages of its direct introduction through a catheter. Such a direct method allows higher drug concentrations, increasing the tissue exposure to the drug, and minimizes systemic side effects. However, the efficacy of this treatment is restricted, due to the urothelium's extremely low permeability as well as urine diluting and washing the drug out. The success of intravesical drug delivery can be improved by prolonging the residence time in the bladder and enhancing adherence and penetration through the urothelium (GuhaSarkar and Banerjee 2010).

It is known that the rate of endocytosis decreases with differentiation stage of UCs (Kreft et al. 2009b), and this could be exploited for differentiation-dependent drug delivery. Increased permeability of the urothelium during different urothelial diseases can actually be taken as an advantage, since the uptake of drugs is thus enhanced. Additionally, it is thought that UP plaques may hinder adhesion and internalization of extracellular substances, which conveys the suitability of unthickened membrane regions as potential sites for biopharmaceutical internalization (Kreft et al. 2009b). On the other hand, UPs are highly glycosylated, and UPIa is a receptor for uropathogenic *Escherichia coli* (UPEC) expressing type 1 pilus adhesion FimH (Wu et al. 1996; Zhou et al. 2001; Xie et al. 2006). There is evidence that UPEC also binds to the heterodimer UPIb/UPIIIa and that UPIIIa is the mediator for the pathogenic cascade and apoptosis, making it a novel therapeutic target (Thumbikat et al. 2009). Based on knowledge gained from numerous studies focused on understanding urothelial biology, many different systems have been developed that aim to improve and optimize intravesical drug delivery.

5.1.1 Glycotargeted Delivery Systems

The fact that UPEC utilizes lectin-mediated cell adhesion to facilitate their internalization leads us to consider glycotargeted drug delivery. Urothelial membrane proteins are highly glycosylated, which could be exploited for selective targeting of cells using lectins (carbohydrate-binding proteins). Lectins show a discrimination similar to antibodies with a high degree of specific interaction (Neutsch et al. 2011). *Wheat* germ *agglutinin* WGA has a high binding potential and a significant preference for malignant tissue, whereas peanut agglutinin PNA has the highest cancer selectivity among lectins, most likely due to deviant glycosylation during carcinogenesis (Neutsch et al. 2011). Another study also demonstrated the selective affinity of the lectins from *Artocarpus integrifolia*, *Amaranthus*

caudatus, and *Datura stramonium* to neoplastic urothelium and thus the implications of lectin-mediated targeted drug delivery (Zupančič et al. 2014a).

5.1.2 Mucoadhesive Biomaterials

Similar to lectins, mucoadhesive biomaterials also strongly adhere to the urothelium, because hydrophilic macromolecules form numerous hydrogen bonds with the GAG layer, allowing the drug to be held at a specific site for prolonged periods and preventing drugs from being washed out. Polymeric hydrogels are also capable of remaining attached on the bladder wall for long duration without being washed. Other treatments involve intravesical thermo-chemotherapy or gene therapy (GuhaSarkar and Banerjee 2010).

5.1.3 Permeability Enhancers

Alternatively, permeation can be increased physically with iontophoresis or electrophoresis, which helps drugs traverse the urothelium. Permeability can also be enhanced chemically with DMSO, protamine sulfate, hyaluronidase, or chitosan hydrochloride (GuhaSarkar and Banerjee 2010). In vitro and in vivo studies have indicated that chitosan hydrochloride is a promising agent in disrupting the urothelial barrier, which increases urothelial permeability in low concentrations and causes desquamation of superficial UCs in high concentrations (Erman et al. 2013; Visnjar and Kreft 2014).

5.1.4 Nanocarriers

Local drug concentrations can be increased with nanocarriers, which can also enhance permeation and target diseased cells. Nanocarriers can be liposomes, solid nanoparticles, protein nanoparticles, polymeric nanoparticles, dendrimers, and magnetic and inorganic nanoparticles (GuhaSarkar and Banerjee 2010). Gold nanoparticles are valuable markers for assessing urine internalization as well as differentiation and functionality of UCs (Hudoklin et al. 2013). Gold nanoparticles have also been studied in conjunction with photothermal therapy for urothelial cancer (Chen et al. 2010) as well as gelatin nanoparticles (Lu et al. 2011). Additionally, studies of mesoporous TiO_2 microbeads and photocatalysis have shown promising results regarding an increased cytotoxicity of urothelial cancer cells (Imani et al. 2014).

6 Conclusions

Overall, the urothelium represents a blood–urine barrier that prevents reabsorption of noxious compounds from urine. This blood–urine barrier is the tightest barrier found in the human body due to its many specialized features, including well-developed tight junctions, UP-containing urothelial plaques, specific lipid compositions of cell membranes, and the luminal glycosaminoglycan layer, which all decrease the amount of permeation that occurs across the urothelium. Much research has been done in the past decades to help our understanding of urothelial biology, and the unique properties of the urothelium must be considered in the development and application of drugs for targeted drug delivery as well as in the development of biomimetic in vitro models and tissue engineering. With further comprehensive knowledge of urothelial function, different alterations of barrier tightness could be achieved that would improve intravesical drug therapies.

Acknowledgments The study was supported by the Slovenian Research Agency (Grant No. P3-0108). The authors thank Professor Dr. Tung-Tien Sun (New York University Medical School) for his generous gift of uroplakin (anti-AUM) antibodies, Dr. Tina Cirman (Blood Transfusion Centre of Slovenia, Ljubljana) for preparing amniotic membranes, and finally various colleagues from the Medical Faculty of the University of Ljubljana for useful discussions.

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