Chapter 9
Epithelial Cell Models; General Introduction

Tor Lea

Abstract An extremely important feature of the intestinal epithelium is its function as a physical barrier between the environment and our bodies' internal milieu. At the same time it has to allow for uptake of important nutrients. At least four different transport mechanisms exist that allow selective uptake and transport of macromolecules across the epithelial cell layer, i.e. paracellular transport, passive diffusion of molecules from the apical to the basolateral side, vesicle-mediated transcytosis and carrier-mediated uptake and diffusion through the epithelial cell layer. Each of these transport mechanisms depends on the physicochemical properties of the compound, its ability to interact with the plasma membrane, its molecular weight and size, stability and charge distribution. In vivo, parameters not directly associated with the molecule in question will influence uptake and transepithelial transport. Intestinal motility, interactions with other molecules from the diet and the digestive process like bile salts and enzymes, and solubility in the mucus layer will affect the absorption process. Thus, in vitro models for studying absorption through the intestinal epithelium have several limitations. Still, they are considered useful model systems for such purposes. Similarly, effects of bioactive molecules on the epithelium can be studied by measuring barrier function and effects on transport processes.

Keywords Polarized epithelium • barrier function • tight junctions • transepithelial transport • paracellular transport • transepithelial electrical resistance (TEER) • permeability coefficient

The one cell-layer thick intestinal epithelium has two challenging and opposing missions. The first is to represent a physical barrier between the contents of the gut lumen and the rest of our body. The second is to ascertain efficient absorption of essential nutrients from the gut lumen and produce mucus with protective properties, anti-microbial peptides that affect microbiota composition, and cytokines with

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both protective and immune-regulatory properties. The small and large intestine are differently organized, have different functional properties and exhibit differences regarding resistance to injury and disease susceptibility.

The small intestinal epithelium is organized into numerous units of crypts and villi. The villi project into the lumen to maximize nutrient breakdown and absorption. It has been estimated that each crypt domain comprises approximately 250 cells, and an equivalent number of cells are generated every day. The epithelial cell layer includes four major cell types that all derive from adult stem cells located at the crypt bottoms: (1) absorptive enterocytes, (2) goblet cells responsible for mucus secretion, (3) enteroendocrine cells that secrete important hormones, and (4) Paneth cells that secrete antimicrobial peptides. In addition, rare and characteristic M-cells (microfold cells) are found in the epithelium overlaying the organized lymphoid tissue, and Peyers patches, in the small intestine. For various purposes, the intestinal epithelium has a very high turnover rate. In the small intestine, apoptotic absorptive enterocytes are continuously sloughed off into the lumen completely renewing the epithelium every 5–7 days.

An extremely important feature of the intestinal epithelium is its function as an efficient barrier between the environment and our bodies' internal milieu. Firstly, the single layer of columnar epithelial cells acts as a mechanical barrier, and, in addition, throughout the whole digestive tract, the epithelium is covered with a highly viscous mucus layer that both trap antimicrobial peptides and neutralizing secretory IgA antibodies. In addition, the mucus layer reduces the direct contact between the luminal microbiota and the epithelial cell layer. The mucus is made up of mucins, i.e. proteoglycans, among which MUC2 dominates in the intestine. There are two types of mucus organization in the intestine. The small intestine has a single mucus layer, while the colon has a two-layered system with an outer and an inner layer (reviewed by Johansson et al. 2013). Despite the high load of microbiota in the colon, the inner mucus layer is more or less impermeable to the lumenal bacteria.

The epithelial cell layer is selectively permeable to bacterial metabolites and digested nutrients allowing regulated transport of soluble molecules through the paracellular space between the epithelial cells. Paracellular transport is controlled by intercellular tight junction (TJ) structures. Disruption of the intestinal TJ barrier, followed by permeation of lumenal noxious molecules, induces a perturbation of the mucosal immune system and inflammation, and can act as a trigger for the development of intestinal and systemic diseases. Over the past decade, there has been increasing recognition of an association between disrupted intestinal barrier function and the development of autoimmune and inflammatory diseases. Recently, although experiments were carried out in *Drosophila*, barrier function turned out to be a better predictor for life expectancy than nominal age and any other clinical parameter tested for Rera et al. (2012).

The protein networks connecting epithelial cells form three adhesive complexes: desmosomes, adherens junctions, and tight junctions (see Fig. 9.1). These complexes consist of transmembrane proteins that interact extracellularly with adjacent cells and intracellularly with adaptor proteins that link to the cytoskeleton. TJ barrier disruption and increased paracellular permeability, followed by permeation of lumenal proinflammatory molecules, can induce activation of the mucosal immune system, resulting

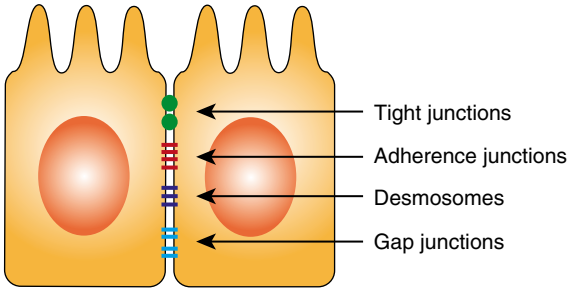

Fig. 9.1 The figure shows a schematic drawing of polarized epithelial cells with different types of intercellular contacts being essential for maintenance of barrier function and communication between neighboring cells

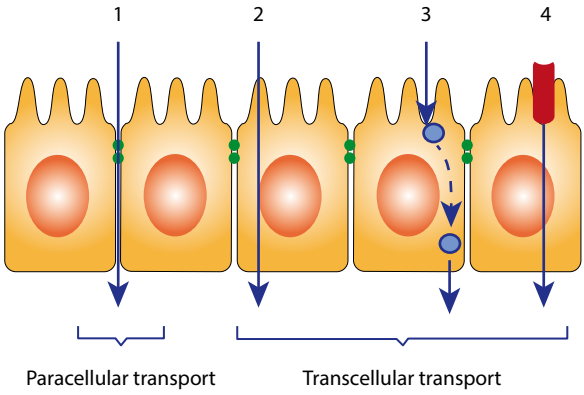

Fig. 9.2 The figure illustrates the different modes of absorption and transport through the intestinal epithelium: (1) paracellular transport, (2) passive diffusion of molecules from the apical to the basolateral side, (3) vesicle-mediated transcytosis and (4) carrier-mediated uptake and diffusion through the epithelial cell layer

in inflammation and tissue damage. Thus reduced barrier function may have far reaching consequences, not only for gut health but also systemic health.

As mentioned above, the intestinal epithelium is not only a mechanical barrier that prevents entry of molecules into circulation. At least four different transport mechanisms exist that allow selective uptake and transport of macromolecules across the epithelial cell layer (Fig. 9.2). Transport of molecules through the epithelium by paracellular transport is regulated by complex intracellular processes finely tuning the permeability of the tight junction complex (1 in Fig. 9.2). Some nutrients or microbial metabolites have properties allowing them to diffuse passively through the enterocyte plasma membrane and through the cell to be released at the basolateral side (2 in Fig. 9.2). Endocytosis (eventually via membrane receptors) allows vesicle-mediated uptake, transport and release at the basolateral side in a process called transcytosis (3 and 4 in Fig. 9.2). Various membrane associated carrier systems may contribute in internalizing luminal molecules allowing further diffusion through the cell cytoplasm and release at the basolateral side.

Each of these transport mechanisms depends on the physicochemical properties of the compound, its ability to interact with and pass the plasma membrane, its molecular weight and size, stability and charge distribution. In vivo parameters not directly associated with the molecule in question will influence uptake and transepithelial transport. Typically, intestinal motility, interactions with other molecules from the diet and the digestive process like bile salts and enzymes, solubility in the mucus layer which increases in thickness from the proximal to the distal part of the

small intestine, will affect the absorption process. Thus, in vitro models for studying absorption through the intestinal epithelium have many limitations. Still, they are considered as useful model systems for such purposes. Similarly, effects of bioactive molecules on the epithelium can be studied by measuring barrier function and influences on transport processes.

Epithelial cells from the intestine have been very difficult to cultivate in vitro as primary cells. Recent technological developments have allowed the growth of intestinal organoids in 3D culture. This approach is described in Chap. 22 of this book. However, 3D organoids are difficult to use for screening purposes. During the last four decades the preferred model of the intestinal epithelium has been transformed, continuously growing epithelial cell lines like Caco-2, and HT29. Both are described in the following chapters. Caco-2 demonstrates many of the properties associated with the enterocytes of the small intestine. When grown on filter supports, after reaching confluence the Caco-2 cells will spontaneously start to differentiate into a polarized cell layer with apical microvilli and intercellular tight junction complexes. Caco-2 cells also express many of the enzyme markers and transport systems of primary epithelial cells. However, Caco-2 cells do not produce mucins, a property present in the HT29 cell line. When HT29 cells were treated with the antimetabolite methotrexate, they differentiated into mature goblet cells (Lesuffleur et al. 1990). Mucus-secreting HT29-MTX cells have been characterized with regard to tight junction formation, development of confluent monolayers and production of the mucus layer. Confluent HT29-MTX cells develop functional tight junction complexes, but not to the same degree as Caco-2 cells. Thus, Caco-2 cells have been more often used to study epithelial barrier function and transepithelial transport than HT29. To compensate for lacking mucus production a co-culture system has been developed based on Caco-2 and the HT29-MTX cell line. This co-culture system is described in detail in Chap. 13. Irrespective of the cell model chosen, e.g. Caco-2, HT29 or Caco-2/HT29 co-cultures, the main readout parameters are effects on the epithelial barrier function, absorption or transepithelial transport of the test compound. The reliability of such studies depends on the uniformity and integrity of the confluent and polarized cell monolayer. Monolayer integrity can be verified in different ways, e.g. by measuring transepithelial electrical resistance or by measuring the passage of the fluorescent dye Lucifer Yellow. Both methods are described below.

9.1 Measurement of Transepithelial Electrical Resistance (TEER)

Determination of transepithelial electrical resistance is a simple and convenient technique that provides information about the uniformity of the Caco-2 cell layer on the filter support, and the integrity of the tight junctions formed between the polarized cells. Thus, TEER measurements are often used to study epithelial barrier function. The electrode is placed in the medium in the upper chamber, and resistance is directly measured by a portable voltmeter like the Millicell-ERS Voltmeter

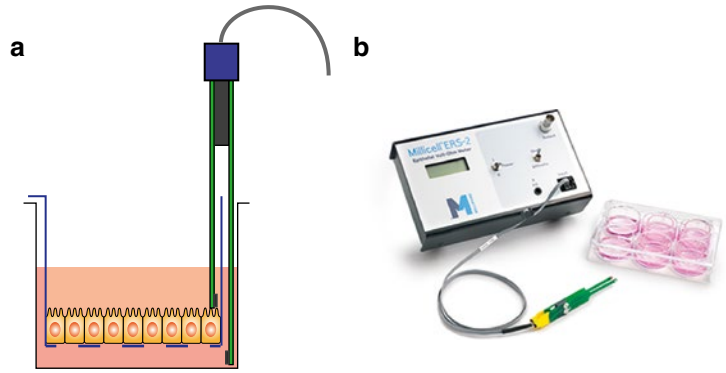

Fig. 9.3 The figure shows The Millicell ERS-2 unit with an STX chopstick electrode. To the left, a drawing of the electrode placed in a tissue culture insert. Note that the shorter tip of the electrode should not be in contact with the cell layer, while the long tip should just touch the bottom of the outer chamber

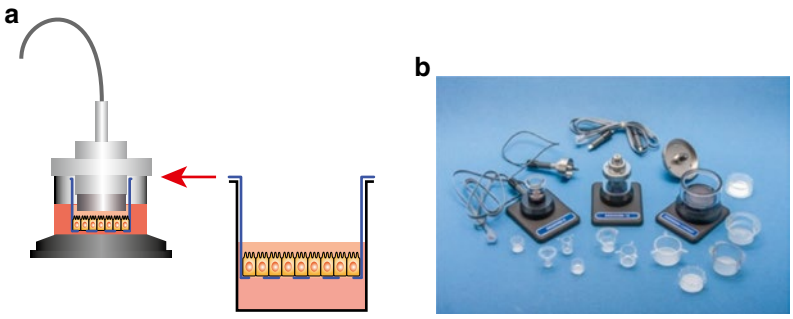

Fig. 9.4 The figure shows different variants of the Endohm chambers with culture inserts for 6, 12 and 24 well plate inserts. To the left is a schematic drawing of the chamber with culture insert. The gap between the upper electrode and the cell layer should be adjusted to 1–2 mm

(Millipore) or the EVOM2, Epithelial Voltohmmeter for TEER (World Precision Instruments Inc., Sarasota, FL) (see Figs. 9.3 and 9.4, respectively).

If the culture is to be continued, the electrode should be sterilized with 70 % ethanol. Before use rinse the electrode in electrolyte solution similar to the culture medium.

9.1.1 Basic Protocol

Measure the resistance of a blank culture cup with no cells that has been thoroughly soaked with an electrolyte solution similar to the culture medium. Move the culture tray from the incubator into the laminar air hood and place it on the heating plate

maintained at 37 °C. Change medium in the culture inserts. Depending on the voltmeter and electrode in use, different protocols have to be followed:

A. With a chopstick probe like the Millicell STX probe, the shorter electrode is placed into the upper compartment (apical side) containing the complete culture medium, and the other electrode in the lower compartment (basolateral side, see Fig. 9.3) containing the same medium. The shorter electrode should not be in contact with the cell layer. Ascertain that the electrode is held steady and at a 90° angle to the plate insert.

   This is perhaps the most common way of measuring TEER. However, the readings with the STX electrodes may vary across the cell layer. An alternative is to use the Endohm electrode system developed for the EVOM2 Epithelial Voltohmmeter (Fig. 9.4) which measures resistance or voltage over the whole area covered by the cells. However, this alternative necessitates transfer of the inserts from their culture wells to the Endohm chamber for measurement rather than using the hand-held STX electrodes. The Endohm chamber and the cap each contain a pair of concentric electrodes, a voltage-sensing silver/silver chloride pellet in the center plus an annular current electrode. The height of the top electrode can be adjusted to fit cell culture inserts from different manufacturers. The circular disc electrodes, situated above and beneath the membrane, allow a more uniform current density to flow across the membrane than with STX electrodes.

B. Sterilize the Endohm chamber and electrode with 70 % ethanol for 15 min and let air dry. Measure the resistance of a blank culture cup with no cells that has been thoroughly soaked with an electrolyte solution similar to the culture medium. Add culture medium to the chamber and transfer the insert to the Endohm chamber and mount the upper electrode. The height of the culture medium outside and inside the insert should be at the same level. Measure resistance in ohms (Ω).

9.1.2 Calculating Transepithelial Resistance

If using a chopstick electrode like the Millicell STX electrode, resistance should be measured several times at different places of a monolayer due to variability in the uniformity of the cell layer, and an average value calculated. The average value of the blank, or preferably blanks, should then be subtracted from the resistance reading of the sample ($R_{sample} - R_{blank} = R_{cell\ layer}$). In a typical example the values can be like: $450\,\Omega - 160\,\Omega = 290\,\Omega$, meaning that the resistance of the monolayer is 290 Ω. Then a correction has to be made for the area covered by the monolayer. Different culture inserts may have different shapes, and the filter covers different areas. Also, data from experiments carried out in different plate formats, i.e. 6-, 12- or 24-well plates must be normalized for comparison. Typically, the area covered by a filter insert for a 24-well plate will be 0.4 cm^2. Multiply the area covered by the cell monolayer with the resistance found in the experiments: $290\,\Omega \times 0.4\,cm^2 = 116\,\Omega\,cm^2$. This value is independent of the area of the membrane used.

9.2 Verification of Monolayer Integrity by Lucifer Yellow Flux

The permeability of the cell monolayer can also be studied by measuring the passive transport of small hydrophilic molecules across the monolayer. Such molecules mainly pass the monolayer via the paracellular route, i.e. through the tight junctions (TJ), and can be used to get information about the leakiness of the TJs. Lucifer Yellow (LY) is an easily detectable marker. It is particularly useful in studies of transepithelial transport of a test compound where it can be used to determine the highest concentration of the sample that does not disturb the integrity of the cell monolayer. The protocol is based on the exposure of the apical side of monolayer to a 4.5 μM solution of LY, eventually together with the test substance, prior to collecting samples from the basolateral side at different time intervals.

9.2.1 Basic Protocol

Prepare the LY (and test compound) solutions immediately prior to each experiment by diluting stocks in buffered Hanks Balanced Salt Solution (HBSS) assay buffer, for example HEPES- or MES-buffered HBSS. LY should be used in a final concentration of 4.5 μM. Prepare a working solution of the test compound containing 4.5 μM LY. Change the medium on the culture inserts 24 h before the experiment. Wash the inserts with buffered HBSS solution to remove medium components. Add 0.5 ml buffered HBSS to the insert compartment (apical) and 1.2 ml to the outside compartment (this is for inserts in a 24-well plate). Keep inserts at 37 °C on a heating plate (temperature is a critical parameter in this type of experiments. Carefully add an extra 0.5 ml LY working solution with or without the test substance to the apical side of the monolayer and 1.2 ml buffered HBSS in the outside compartment (basolateral side). Place the culture trays with inserts on a rotary plate shaker (200–300 rpm at 37 °C). At different time points, for example 30, 60, 90, 120 min, remove a 0.5 ml sample from the basolateral side and replace with 0.5 ml prewarmed buffered HBSS solution. Based on fluorimetric readings (excitation wavelength 428 nm and emission wavelength 536 nm), measure the concentration of LY in the test samples and make a standard curve. Calculate the apparent permeability coefficient (P_{app}) values of the LY flux through the monolayer. P_{app}, (unit: cm s^{-1}) is determined from the amount of compound transported per time by the equation:

$$P_{app} = \left(dQ / dt\right)\left(1 / \left(AC_0\right)\right)$$

where dQ/dt is the steady-state flux (μmol s^{-1}), A is the surface area of the filter (cm^2) and C_0 is the initial concentration in the donor chamber (μM). If the value deviates from the normal values obtained, the cell monolayer integrity is disrupted.

In the same way, the effects of a given compound on epithelial LY permeability can be tested by including the test compound with the LY solution in the donor compartment. Then the calculated permeability coefficient to the test compound (P_{appt})

can be compared with the apparent permeability value of the control cells, e.g. without added compound (P_{appc}). Values of $P_{appt} \leq P_{appc}$ suggest that the test compound can be regarded as non-toxic and that it can be used to perform studies of effects on epithelial barrier function or absorption experiments.

9.3 Summary

The single layer of epithelial cells in the intestine represents the rate-limiting barrier for transport and uptake of compounds between the lumen and the body interior. Thus, in vitro-differentiated human epithelial cell monolayers allows for testing of absorption and active and passive transport processes through the intestinal epithelium. Among available epithelial cell lines Caco-2 has been found particularly suitable. In food science, problems of interest are not only related to absorption and transport issues, but also to biological effects of dietary compounds that may influence other aspects of epithelial function, typically tight junction structure, pattern recognition receptor activation and intracellular signaling processes that may affect cytokine production and subsequently both innate and adaptive immune responses. In such situations the lack of mucus production by Caco-2 cells may be considered a disadvantage. The HT29-MTX cell line may then represent an alternative, or a co-culture of Caco-2 and HT29-MTX cells, that also may provide information about effects on mucus production, an essential part of the intestinal barrier function. All these culture systems are described in the following chapters.

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