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Research paper

Tight junctions and tight junction proteins in mammalian epidermis

Johanna M. Brandner *

Department of Dermatology and Venerology, University Hospital Hamburg-Eppendorf, Hamburg, Germany

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ABSTRACT

Tight junctions (TJ) are barrier forming cell–cell junctions that are found in a variety of cell types and tissues but their existence in mammalian epidermis has been shown only in the last years. A variety of TJ proteins were identified in mammalian epidermis, comprising several members of the claudin family, occludin, and JAM-A as well as ZO-1 and MUPP-1. TJ proteins exhibit complex expression and localization patterns in the epidermis. Nonetheless, even though several TJ proteins are found in various layers, typical TJ structures are only found in the *stratum granulosum*. TJ are important for barrier function of the skin, especially for inside–out barrier. In addition, TJ proteins might also be involved in additional functions in epidermal cells. Localization and expression of TJ proteins are altered in several skin diseases, e.g. psoriasis. Meanwhile several TJ modulators are known from simple epithelia. We discuss their putative usability for drug delivery into and through the skin.

1. Introduction (TJ in simple epithelia and endothelia)

Tight junctions (TJ) are cell–cell junctions that connect neighbouring cells closely. They are very complex structures that are formed by various TJ transmembrane proteins, e.g. the family of claudins (Cldns), occludin and the family of junctional adhesion molecules (JAMs), as well as TJ plaque proteins, e.g. the MAGUK proteins ZO-1, ZO-2, ZO-3, cingulin, symplekin and the cell polarity complex proteins aPKC, Par3 and Par6. TJ structures and/or TJ proteins control the paracellular pathway of molecules ("barrier function") including the paracellular migration of inflammatory cells. Furthermore, they separate the lipids of the apical from the basolateral portions of the plasma membrane which results in the establishment of two different membrane compartments ("fence function"). Consequently, they establish cell polarity for lipids (for reviews see [1–3]). In addition, TJ structures and/or TJ proteins fulfill a variety of further functions, e.g. they are contact sites for molecules of signal transduction pathways and cell surface receptors, e.g. TGFβ receptor, they are involved in cell proliferation and differentiation and they contribute to vesicle transport (for reviews see [1,4]). Precise function of TJ depends on their composition which, in turn, depends on cell type and differentiation as well as on physiological and pathological stimuli. For example, combination and mixing ratio of the proteins of the Cldn family, which comprises 24 members in vertebrates, is, among others, essential for TJ permeability and ion selectivity [5,6].

Several TJ associated proteins are not restricted to TJ but are also found in the cell nucleus, e.g. ZONAB, c-jun and c-fos, which are known to be transcription factors, symplekin, which is associated with the polyadenylation machinery, and ZO-1, ZO-2, and ZO-3 which are known to play scaffolding functions at TJ, whereas their role in the cell nucleus is not completely clear. ZO-1 has also been identified at adherens junctions and gap junctions (for reviews see [1,2,4,7,8]).

Mutations in genes coding for TJ proteins are the origin for several inherited diseases, e.g. NISCH syndrome (neonatal sclerosing cholangitis associated with ichthyosis) which is caused by mutations in the gene coding for Cldn-1, non-syndromic deafness which results from mutations in the gene coding for Cldn-14, and hypomagnesaemia with hypercalciuria and nephrocalcinosis which originates from mutations in the gene coding for Cldn-16/paracellin1 (for reviews see [9,10]). In addition, alterations of TJ proteins and structures are found in several other ailments, e.g. in inflammatory diseases, such as Morbus Crohn and acute lung inflammation [11,12] as well as in various tumors (for review see [13]).

TJ proteins are known to be targets for bacterial, viral and allergic insults. For example, the enterotoxin of *Clostridium perfringens*, which causes severe diarrhoea, binds directly to Cldn-3 and -4 – originally identified as "*Clostridium perfringens* enterotoxin-receptors – and separates Cldn-4 from TJ [14,15]. Still, the importance of binding to Cldn-3 and -4 for the clinical symptoms is not clear, as the NH2-teminal region of the enterotoxin increases membrane permeability by forming small pores [16,17]. Besides direct inter-

* Corresponding author. Department of Dermatology and Venerology, University Hospital Hamburg-Eppendorf, Martinistrasse 52, 20246 Hamburg, Germany. Tel.: +49 40 42803 5819; fax: +49 40 42803 2655.
E-mail address: brandner@uke.uni-hamburg.de.

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actions, TJ can also be altered secondarily during bacterial infection, e.g. via signal transduction pathways and alterations of the actin filament cytoskeleton (for reviews see [18,19]). Viruses have been described to use TJ proteins as (co)receptors, e.g. JAM-A is used by reovirus and Cldn-1 by hepatitis C virus [20,21]. Allergens with proteolytic activity, e.g. Der p-1, a cystein-protease of the house dust mite, can disrupt TJ and might thereby facilitate the accessibility of epithelia to the allergens and be important for the development of asthma (for reviews see [1,2,18,22]).

2. TJ proteins and structures in the epidermis

The existence of TJ in mammalian epidermis has – after decades of discussion [23–32] – finally been demonstrated in both human and mouse epidermis at the beginning of this century [33–36]. The successful identification of TJ structures is ascribable to the use of antibodies recognizing TJ proteins as markers, allowing a more specific search for the structures. Previous reports questioning the existence of TJ structures mostly did not have the benefit of these antibodies for their studies.

Up to now a variety of TJ proteins have been identified in mammalian epidermis by using specific antibodies, i.e. Cldn-1, Cldn-3 (faintly), Cldn-4, Cldn-5 (faintly), Cldn-7, occludin, JAM-A, cingulin, ZO-1, Mupp-1, and symplekin, in human and additionally Cldn-6, Cldn-11, Cldn-12 and Cldn-18, ZO-2, aPKC, Par3, and Par6 in mouse epidermis (investigation of most of these proteins has not been done as yet in human skin but some of the proteins have already been identified in human cultured keratinocytes) [23,24,34,36–42]. Additional TJ molecules, e.g. Cldn-8 and Cldn-17, have been identified on mRNA level in human keratinocytes. The distribution patterns of the various TJ proteins in the epidermis are diverse. While, e.g. occludin and cingulin are restricted to the *stratum granulosum* (SG), some proteins, e.g. ZO-1 and Cldn-4 are found in several suprabasal layers, and other proteins, e.g. Cldn-1 and MUPP-1 are localized in all epidermal layers (Fig. 1).

Typical TJ structures comparable to those known from simple epithelia, i.e. seen in transmission electron microscopy as small subapical regions of direct contact between the plasma membranes of two adjacent cells without extracellular gaps or intermembranous material (“kissing points” or “sites of fusion”) (Fig. 2) are found in the lateral plasma membranes of the keratinocytes in the SG of human and mouse. This is also the area, where (1) all TJ proteins present in the epidermis colocalize, (2) apparently continuous zonula occludens-like immunostainings in horizontal sections are found [34,43], and (3) the extracellular diffusion of an intradermal injected tracer of 600 Da stops, hinting for functionality of these structures as a barrier (Fig. 1) [35,41,44]. The latter has up to now only been published in mouse skin. The typical TJ structures found in the epidermis are often interspersed with desmosomes [34,35,43]. As several TJ proteins also colocalize in deeper layers of the epidermis, additional TJ-related structures with putatively different barrier properties, might be postulated, but have not been shown as yet. In various stratified epithelia, e.g. bovine gingiva and muzzle epithelium, other structures containing occludin have been identified, including occludens junctions, lamellated junctions (coniunctiones laminosae) and sandwich junctions (iuncturae structae) [43]. The function of these structures is so far unknown.

A network of anastomosing fibrils which is typical for TJ structures in freeze-fracture electron microscopy in simple epithelia and endothelia has been found in vitamin-A or humid milieu treated chicken and mouse epidermis [27,28], cultured human skin keratinocytes [32], and occasionally in human fetal skin [30]. In addition, structures similar to these have been identified at the border between SG and *stratum corneum* (SC) in human epidermis [45]. However, in the SG of adult untreated skin these structures have not been identified as yet. The difficulties in identification may be due to their restricted localization in mammalian skin.

In addition to keratinocytes, Cldn-1 has also been identified in stationary and migratory Langerhans cells [46].

TJ proteins have also been identified in human and mouse hair follicles and in human cutaneous glands [43,47,48]. This argues for a continuous TJ system in the skin, but typical TJ structures or TJ-related structures in skin appendages have not been shown as yet.

3. Dynamics of TJ in the epidermis and in cultured keratinocytes

In cultured keratinocytes it has been shown that the formation of (functional) TJ depends on Ca-concentration. Ca^{2+} induced differentiation of human and mouse keratinocytes is accompanied by a continuous localization of TJ proteins at the cell–cell borders [34,36,38,49,50] and by the establishment of transepithelial resistance (TER), as well as by decreased permeability for molecular tracers, both measures for TJ tightness [38,49,50]. Ca^{2+} depletion results in a loss of TJ proteins from the cell–cell borders and a decrease in TER [50]. Inhibition of aPKC which is associated with TJ as part of the cell polarity complex Par3/Par6/aPKC abolishes the establishment of TER after Ca-induced differentiation of mouse keratinocytes; overexpression of aPKC accelerates TER formation [38].

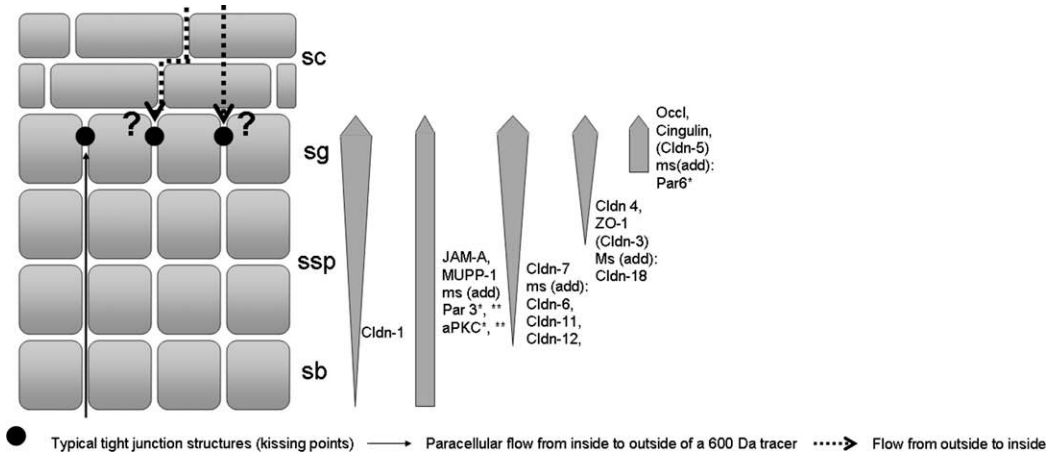

Fig. 1. Schematic drawing of the epidermis showing localization of typical TJ structures as well as of TJ proteins. *Different localizations of various isoforms; **partly in the cytoplasm and at cell–cell borders; sb, stratum basale; ssp, stratum spinosum; sg, stratum granulosum; sc, stratum corneum; ms, mouse, add, additional. Proteins mentioned in brackets have been observed only very faintly.

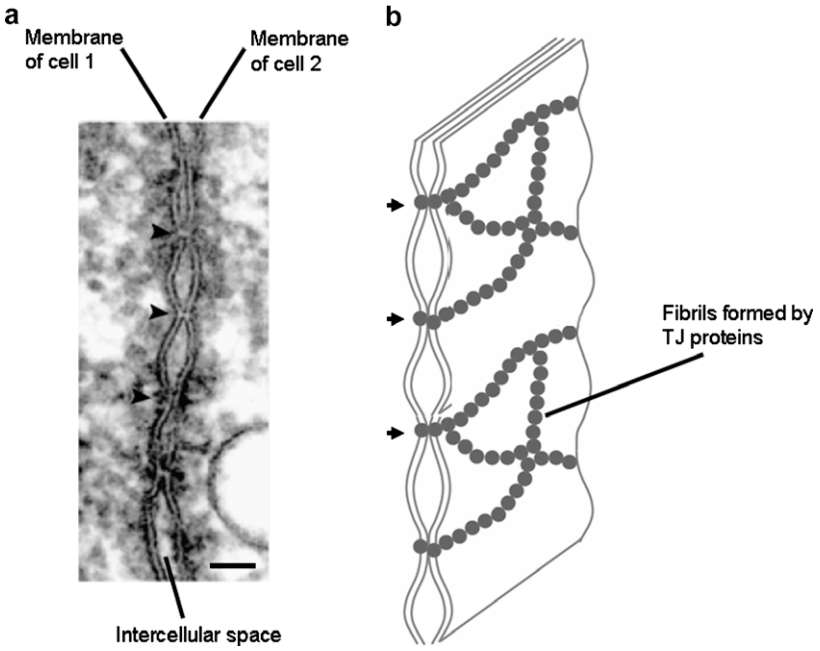

Fig. 2. (a) Transmission electron-microscopical image showing typical TJ structures (kissing points; arrowheads) in intestine epithelial cells. Bar, 50 nm. (b) Schematic drawing of membranes of neighbouring cells and the connecting TJ. TJ proteins from the apposing membranes contact each other and form structures which obliterate the intercellular space. Modification of Fig. 2 in [75].

Tiam-1 (T-lymphoma invasion and metastasis), an exchange factor for the small GTPase Rac, plays an important role in the formation of functional TJ in mouse keratinocytes. It directly regulates TJ assembly through the activation of the par polarity complex. Tiam-1 deficient keratinocytes form normal primordial adhesions that contain components of both AJ and TJ and are properly localized but they are impaired in TJ maturation. Tiam-1 triggers the biogenesis of the TJ through the activation of Rac and aPKC independently from Cdc42 [49].

A colocalization of AJ and TJ proteins is observed in the uppermost spinous and lower granular cell layers of human epidermis and at the border of companion cell layer and Henlés layer in the lower portion of human hair follicles [36,47]. It is possible that the areas with colocalizing TJ and AJ proteins in the epidermis and hair follicles represent the primordial adhesions mentioned above. However, it cannot be excluded that AJ and TJ are so tightly grouped together in these areas that they cannot be separated on the light microscopic level. Alternatively, a special skin structure containing TJ and AJ proteins might exist.

4. Involvement of TJ in barrier function of the epidermis

Although SC, the outermost layer of the epidermis, is recognized as the most important physical barrier, the lower epidermal layers are also significant in barrier function. Loss of nucleated epidermal layers through the induction of suction blisters results in a tremendous transepidermal water loss (TEWL), which is much higher than TEWL observed after the removal of SC by tape stripping. Also the loss of the epidermis over large areas of the body as seen in patients with burn injury or extensive *Pemphigus vulgaris* – who may die because of extensive water loss or sepsis induced by external bacterial infection – is much more severe than the loss of SC only.

When talking about barrier function of the epidermis, one has to distinguish between inside–out barrier, i.e. prevention of the loss of water and solutes, and outside–in barrier, i.e. protection against the entry of harmful substances, such as microbes and chemicals.

4.1. Inside–out barrier

TJ play an important role in inside–out barrier of the skin. As early as 1971, Hashimoto showed that the electron dense tracer lanthanum stopped at TJ in the SG of human epidermis [31] but these results could not be verified by others [26,28]. About 30 years later, Cldn-1 deficient-mice confirmed the role of TJ in inside–out barrier. These mice are characterized by a tremendous TEWL and die within one day of birth due to the dehydration [35]. No morphological abnormities in the viable epidermal layers and TJ structures on light- and electron-microscopic levels have been found in these mice but a permeability assay using an intradermal injected 600 Da tracer revealed leaky TJ in the SG. SC was thicker and more compact but its composition seemed unaltered. Increased TEWL and leaky TJ were also observed in epidermal E-cadherin knock-out mice. Interestingly, deficiency for E-cadherin results in a loss of Cldn-1 from the SG and an altered distribution of further TJ proteins [41]. Still, it has not been clarified as yet, whether leaky TJ are the primary cause for the elevated TEWL in the ko-mice due to an increased diffusion of water through TJ, or whether it is a secondary effect due to disturbance of TJ-dependant ion gradients in the epidermis which might change synthesis and processing of proteins and lipids involved in SC barrier function. However, both explanations show the importance of TJ for inside–out barrier function of the epidermis, at least in mice. Amazingly, it has not been demonstrated as yet that water is able to pass through TJ pores, even though it has long been stated, e.g. in the solvent drag model [51]. Kovbasnjuk and colleagues even described that there is no water flow through TJ of cultured kidney epithelial cells [52], but this might be a phenomenon of special cell types. Another explanation for the phenotype seen in Cldn-1 and E-Cadherin ko-mice might be a loss of TJ fence function and therefore a change in cell polarity for lipids abolishing the directed secretion of lamellar bodies, and leading to impaired SC function.

A loss of inside–out barrier function and leaky TJ were also observed in mice deficient for CAP1/Prss8 (a serine protease), which are characterized by the absence of occludin from SG [44]. How-

ever, as occludin deficient-mice do not exhibit an evident alteration of epidermal barrier function [own observations and 53] and as CAP1/Prss8 deficient-mice also show various changes in SC, occludin deficiency might not be the primary cause for the phenotype.

Mice overexpressing Cldn-6 in the epidermis also show elevated TEWL and die soon after birth [54]. This denotes that loss as well as overexpression of TJ proteins in the epidermis results in a disturbance of inside-out barrier of mouse skin. Therefore, the exact composition of epidermal TJ seems to be crucial for their function.

Even though these experiments performed in mice are very convincing, a confirmation of the importance of TJ for inside–out barrier function in human skin is indispensable.

4.2. Outside–in barrier

In contrast to the inside–out barrier, it is as yet inexplicit whether TJ are involved in outside-in barrier of the skin. Experiments elucidating outside–in dye penetration of Cldn-1 deficient-mice have not been published; E-Cadherin ko-mice do not show any alteration in outside–in permeability [41]. On the other hand, CAP1/Prss8 deficient-mice as well as Cldn-6 overexpressing mice are characterized by increased outside–in dye penetration [44,54]. However, these mice also show changes in SC morphology as well as in SC lipid composition or expression of late epidermal differentiation markers. Mice deficient for the Grainyhead-like epithelial transactivator (Get-1) which show a mislocalization and down-regulation of TJ proteins also show an impairment of outside–in barrier function. Nonetheless, the deficiency for this transcription factor also leads to, among others, alterations in epidermal differentiation and extracellular lipid composition [55].

Summarizing these observations one might speculate that an impairment of outside–in barrier arises only when TJ-deficiency leads to an alteration of SC composition. Nonetheless, TJ might be second line of defense from outside to inside when SC function is impaired or when SC has been conquered.

5. Additional functions of TJ proteins in the epidermis

In simple epithelia and endothelia, TJ proteins have also been described to be involved in additional functions, e.g. in cell differentiation and proliferation, in vesicle transport and in fence function for membrane lipids. As already mentioned above, alterations seen in Cldn-1 and E-Cadherin ko-mice might also, at least partly, be explained by impaired cell polarity for lipids in the SG. However, this has not been shown as yet. Interestingly, Cldn-6 overexpressing mice show alterations in differentiation and proliferation of epidermis and skin appendages [56]. Consequently, TJ proteins might also in the skin fulfill functions additional to barrier function.

ZO-1 seems to be involved in non-barrier related functions too. While this protein is absent in epidermal melanocytes, an up-regulation is observed in human melanoma cells that seems to be connected to invasiveness of the cells. However, ZO-1 in melanoma cells seems to be associated with adherens junctions [57].

6. Epidermal TJ proteins in skin diseases

NISCH syndrome (neonatal sclerosing cholangitis associated with ichthyosis) is the only skin disease caused by mutations in a TJ protein, i.e. Cldn-1, that has been identified so far. Patients suffering from NISCH syndrome exhibit white scaly ichthyosis, hypotrychosis/alopecia and eyelashes/eyebrows abnormalities [58]. Number and distribution of Langerhans cells is not altered [59].

In various human skin diseases, altered expression of TJ proteins has been observed. *Psoriasis vulgaris*, *Ichthyosis vulgaris* and *lichen ruber planus* are characterized by a broadened localization of TJ proteins that are normally restricted to the upper layers of the epidermis, i.e. Cldn-4, occludin, and ZO-1 [24,36,37,40]. Perilesional skin of patients suffering from psoriasis shows largely normal distribution of these TJ proteins, healed psoriatic plaques a re-established localization for all proteins except for Cldn-4 ([40]). A broadened expression of TJ proteins is also observed in wounded human skin at the wound margins and in the regenerating epidermis, a skin condition where SC barrier is absent [34,60]. In addition, an up-regulation of TJ proteins is observed in porcine and human skin colonized with non-pathogenic and, to some extent, with pathogenic bacteria [39]. Interestingly, this broadened expression resembles the expression of TJ proteins in stratified epithelia bordering on moist surroundings, for example in gingival, exocervical and vaginal epithelia [43], and in immature epidermis in the course of formation of skin equivalents [34]. It also reminds of the increased number of TJ strands in freeze-fracture electron microscopy observed in Vitamin-A-treated chicken skin, as well as in mouse skin incubated in humid environment [27,28]. Therefore, a broadened expression of TJ proteins seems to be observed when SC barrier is altered, absent or challenged. Further experiments elucidating the function of this broadened expression have to be performed.

Whereas the formerly restricted TJ proteins show a broadened expression in psoriasis as well as in other skin diseases, Cldn-1, which is normally expressed in all layers of the epidermis, is down-regulated in psoriatic epidermis, especially in the lower layers [37,42]. This might be due to the production of IL1ß, which has been shown to downregulate Cldn-1 in epidermis [42]. A down-regulation of Cldn-1 (as well as of Cldn-6, -11, -12 and -18) in lower layers of the epidermis was also observed in a mouse skin squamous cell carcinoma tumorigenesis model. [61]. As psoriasis as well as the tumorigenesis model is characterized by the infiltration of inflammatory cells, one might speculate that this might lead to the alterations of Cldn expression. In human squamous cell carcinoma it has been shown that TJ proteins Occl and Cldn-4 are restricted to certain areas, while Cldn-1 shows a more widespread but very variable distribution [62,63]. Interestingly, TJ proteins are co-expressed in squamous cell carcinomas in areas that do not border to lumina or body surfaces. One putative explanation is that they separate some tumor areas from external influences, e.g. the immune system and cytostatic drugs.

During human and porcine skin infection by *Staphylococcus aureus* a down-regulation of TJ proteins in the upper layers and subsequently also the lower layers of the epidermis is observed. In addition, a loss of TJ function in cultured human keratinocytes was demonstrated [39]. This suggests that TJ might play a role as a barrier for pathogen invasion.

7. Potential usability of TJ for (trans)cutaneous drug delivery

Permeation of topically applied drugs through the epidermis is an important issue, both for drug delivery into the skin and for systemic drug delivery. As the epidermis is specialized in avoiding its penetration by external substances, the barrier function of the skin has to be overcome by permeation enhancers. Ideal properties of permeation enhancers would comprise their compatibility with the drug and other formulation excipients and its immediate enhancing of permeation. In addition it should act reversibly, it should reduce barrier function in one direction only, should be pharmacologically inert, non-toxic, and, last but not least, inexpensive. In general it is known that drug delivery could follow a transcellular as well as a paracellular route.

For the epidermis, the first barrier that has to be overcome is the SC. Its role in drug delivery is discussed elsewhere (e.g. [64]). Whether an additional opening of TJ for the attenuation of paracellular outside-in barrier and therefore for drug delivery in healthy skin is necessary (see also chapter 3) and whether the permeation enhancers currently used influence epidermal TJ is not clear at the moment.

TJ might be especially important for drug delivery in skin with impaired SC barrier function, e.g. in psoriasis, where several TJ proteins are up-regulated. This might specifically apply for hydrophilic substances that use the paracellular pathway. In addition, as described above, modulation of TJ might lead to a change in SC permeability, suggesting that this could be a very subtle tool for the alteration of (SC) skin permeation.

From simple epithelia we know that solutes with a molecular radius exceeding 15 A (ca. 3.5 kDa) might be excluded from the paracellular uptake route [65]. TJ modulators have been investigated as permeation enhancers since the 1960 s. Several substances which enhance drug delivery through TJ of mucosal epithelia, especially in the intestine, have been described.

TJ modulators (TJM) can be divided into first generation and second generation modulators (for review see [66]). First generation TJM do not target specifically TJ components but open TJ via secondary effects, e.g. EDTA, which depletes extracellular Ca^{2+}, leading to a disruption of TJ via protein kinase C [67,68], and sodium caprate, which targets phopholipase C, resulting in increased intracellular Ca^{2+} levels, which lead, via an activation of myosin light chain kinase, to the contraction of actin filaments and an opening of TJ [69]. Also several polymers, e.g. chitosan and carboxymethylcellulose, belong to the first generation TJM. They result in an activation of tyrosine kinases and a modification of occludin which leads to increased TJ permeability [69,70]. In addition, several other molecules have been shown to influence TJ, e.g. NO, acyl carnithine, salicylates, and 18-beta glycyrrhetinic acid (for reviews see [66,71]). The underlying mechanisms are mostly not completely clear and none of these molecules has as yet been tested for opening TJ in the epidermis. First generation TJM often exhibit low tissue specificity as their target molecules are widely expressed, they are not able to avoid the influx of toxic substances concurrent with the drug, and often cell membrane damage may occur in addition to the opening of TJ. Therefore, they are prone to exhibit side effects.

Second generation TJM target specific TJ components, e.g. the C-terminal (non-cytotoxic) part of *C. perfringens* enterotoxin which specifically binds to the second extracellular loop domain of Cldn-4 and results in a decrease of Cldn-4 levels and reduced TJ integrity [14,15] or peptides specific for the extracellular domains of occludin which also lead to an increase of TJ permeability (for review see [66]). Utilisation of the C-terminal domain of Cldn-4 for absorption enhancement in rat intestine showed good results without evident side effects [72]. A highly specific modulator of TJ is also zonula occludens toxin (ZOT) from *Vibrio cholerae*. ZOT mimics zonulin, an endogenous modulator of intestinal TJ that induces TJ disassembly (for review see [73]). It binds to ZOT receptors which have as yet been identified in small intestine, nasal epithelium, heart and brain endothelium (for review see [71]). It has been shown that Zot can increase TJ permeability quickly and reversibly without affecting the transcellular pathway and without exhibiting toxicity. In general, specific TJM should exhibit less side effects than the first generation TJM, but, again, none of these molecules has been used for the alteration of TJ in the epidermis as yet.

Nonetheless, specific TJ modulators might be promising tools for drug delivery through the epidermis. Cldn-4 as well as occludin has been shown to be present in the upper layers of the epidermis. For zonulin or Zot-receptors no reports have been published concerning their existence in the skin. Further experiments have to clarify the suitability of these modulators as well as of newly developed substances, e.g. specific interfering RNA sequences, for drug delivery into and through the skin. Important aspects that have to be considered are the development of feasible formulations for topical application, avoidance of interactions with the drugs to be delivered, and circumvention of side effects.

Investigation of the usability of the TJM for the opening of TJ in the skin could be performed by penetration studies combined with immunohistological localization of TJ proteins in mouse models and keratinocyte cultures which both have been shown to exhibit functional TJ (e.g. [35,50]). Our own results also show the functionality of TJ in porcine *ex vivo* skin models (unpublished data). TJ proteins are very similar between human, pig and mouse in sequence (e.g. for Cldn-1 93% identity and 100% homology between human and pig as well as 89% identity and 97% homology between human and mouse) as well as in distribution [37,74], therefore a transfer of results to human TJ should be possible. In addition, investigation of *ex vivo* human skin models should be feasible. As up to now TJ cannot be detected by *in vivo* methods, investigation of *in vivo* human skin is not possible. Future studies have to clarify the usability of TJM in skin as well as the ideal test system for their investigation.

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