Summary. Tricellulin is the first molecular component of tricellular tight junctions at tricellular contacts where three epithelial cells meet, and it is required for the their formation and maintenance of the epithelial barrier. Tricellulin binds other tight junction proteins, and its expression and distribution are affected by the bicellular tight junction protein occludin and lipolysis-stimulated lipoprotein receptor (LSR) which is expressed at tricellular contacts. Tricellulin is also detected in endothelial cells, neurons, microglia and astrocytes. Here, we focused tricellulin expression in various types of epithelial cells, nasal epithelial cells, pancreatic duct epithelial cells and hepatocytes, and non-epithelial cells, dendritic cells and Schwann cells, compared to expression of the bicellular tight junction protein occludin and LSR, and discuss the regulation and the role of tricellulin in cellular specificity.

Key words: Tricellular tight junction, Tricellulin, LSR, Pancreatic duct epithelial cells, Dendritic cells, Schwann cells, Hepatocytes

Introduction

The tricellular tight junction forms at the convergence of bicellular tight junctions where three epithelial cells meet in polarized epithelia, and it is required for the maintenance of the transepithelial barrier (Ikenouchi et al., 2005).

Tricellulin, occludin and marvelD3 form the tight junction-associated marvel protein (TAMP) family, based on their sequence homology within the shared MARVEL domain (MAL and related proteins for vesicle trafficking and membrane link) (Raleigh et al., 2010) and it was identified as the first marker of the tricellular tight junction in epithelial cells (Ikenouchi et al., 2005). Tricellulin directly binds to marvelD3 and ZO-1 (Ikenouchi et al., 2008; Raleigh et al., 2010). It has been reported that tricellulin forms a barrier in tricellular tight junctions effective for macromolecules and in bicellular tight junctions for solutes of all sizes (Krug et al., 2009). Human tricellulin mutations are associated with nonsyndromic hearing loss (Riazuddin et al., 2006; Chishti et al., 2008).

The C-terminus of tricellulin exhibits homology to the C-terminus of occludin and is important for the basolateral translocation of tricellulin (Ikenouchi et al., 2005; Westphal et al., 2010). Knockdown of occludin causes mislocalization of tricellulin to bicellular tight junctions (Ikenouchi et al., 2008). Cotransfection of tricellulin and claudin-1 changes the tight junction strand network (Cording et al., 2013). Furthermore, it is known that tricellulin mRNA is detected in endothelial cells, neurons, microglia and astrocytes (Mariano et al., 2011, 2013).

More recently, lipolysis-stimulated lipoprotein receptor (LSR) was identified as a novel molecular constituent of tricellular contacts (Masuda et al., 2011). LSR assembles at the corners of epithelial cells to generate a landmark for tricellular tight junction formation, and tricellulin is recruited to tricellular contacts via its interaction with LSR (Masuda et al.,
Tricellulin in epithelial cells and non-epithelial cells

In freeze-fracture replicas, tight junction strands first appear in regions where three cells meet in differentiating olfactory epithelium and then they are observed in bicellular tight junctions as well as tricellular tight junctions (Mencoc, 1988). Although it is considered to be important for the mucosal barrier of the upper respiratory tract, little is known about the expression and localization of tricellulin.

We investigated the expression and localization of tricellulin in normal human nasal epithelial cells in vivo and in vitro. Both in vivo and in vitro, mRNA and protein of tricellulin were detected (Okuni et al., 2009). It was localized not only at tricellular contacts but also at bicellular borders, and in part colocalized with occludin (Fig. 1A,B). Immunoelectron microscopy analysis revealed tricellulin-associated gold particles around the junction-like structure of the uppermost region (Fig. 1C). In human nasal epithelial cells in vitro, treatment with the PPAR-agonist rosiglitazone induces tricellulin as well as claudin-1 and -4 and occludin with upregulation of the barrier function (Ogasawara et al., 2010).

On the other hand, Rescigno et al. discovered a new mechanism for pathogen uptake in the mucosa by which dendritic cells (DCs) open the tight junctions between epithelial cells and send dendrites outside the epithelium to directly sample the pathogen. DCs express tight junction proteins such as occludin, claudin-1 and ZO-1 to preserve the integrity of the epithelial barrier (Rescigno et al. 2001). Furthermore, activated Langerhans cells gain access to acquisition of external antigens by sending their dendrites out through epidermal claudin-dependent bicellular and tricellular-dependent tricellular tight junctions at Langerhans cells-keratinocyte cells contacts (Kubo et al., 2009).

We previously reported that HLA-DR- and CD11c-positive DCs expressed claudin-1 and penetrated beyond occludin in the epithelium of the nasal mucosa with allergic rhinitis (Takano et al., 2005). When we investigated the relationship between expression of tricellulin and LSR in the epithelium of the nasal mucosa with allergic rhinitis and CD11c-positive DCs, the DCs penetrated beyond tricellulin and LSR, which were expressed in the epithelium (Fig. 1D). It is possible that DCs send dendrites outside the epithelium via tricellular junctions to directly sample pathogens in the human nasal mucosa.

Tricellulin in human nasal mucosa

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Tricellulin in human dendritic cells

In XS52 DCs established from the epidermis of a newborn mouse, tight junction molecules claudin-1, -3, -4, -6, -7, -8, and occludin are detected and expression of all these tight junction molecules is increased after treatment with thymic stromal lymphopoietin (TSLP), which is an IL-7-like cytokine that triggers DC-mediated Th2-type inflammatory responses (Kamekura et al., 2010). Tricellulin mRNA was detected, as were occludin, JAM-A, ZO-1, ZO-2 and claudin-4, -7, -8, and -9 in mature human DCs differentiated from the human monocytic cell line THP-1 by treatment with IL-4, GM-CSF and TNF-α (Fig. 2) (Ogasawara et al., 2009). However, the mechanisms of regulation of tricellulin expression in mature human DCs remain unknown.

Regulation of tricellulin in human pancreatic duct epithelial cells

Tricellulin is localized apically in normal pancreatic ducts, with spotty immunopositivity at tricellular contacts, whereas weaker signals are observed at the junctions between two cells (Korompay et al., 2012).

In hTERT-transfected HPDE (hTERT-HPDE) cells, which are positive for the pancreatic duct epithelial markers CK7, CK19, and carbonic anhydrase isozyme 2, as in normal human pancreatic duct epithelial cells, tricellulin is detected, as are occludin, JAM-A, ZO-1, ZO-2, claudin-1, -4, -7, -8, and -9 (Yamaguchi et al., 2010).

To investigate the mechanisms of regulation of tricellulin in human pancreatic duct epithelial cells, the expression and localization of tricellulin were examined not only in normal human pancreatic duct epithelial cells but also in various human pancreatic cancer cell lines by using Western blotting and immunostaining. In Western blots of all pancreatic cancer cell lines examined, tricellulin was detected and the expression was more strongly observed in HPAC cells than in other cell lines (Kojima et al., 2010). In immunostaining of HPAC cells, tricellulin-positive spots were clearly observed at the tricellular contacts and colocalized with occludin (Fig. 3A), whereas TRIC immunoreactivity was observed not only at the tricellular contacts but also at the bicellular borders in other cell lines. In HPAC cells, LSR was strongly expressed at the tricellular contacts and was also colocalized with occludin (Fig. 3A). In immunoelectron microscopy, tricellulin-associated gold particles are observed near the tricellular contacts (Fig. 3C).

On the other hand, it is known that tight junction proteins are regulated by various cytokines and growth factors via distinct signal transduction pathways (González-Mariscal et al., 2008). Protein kinase C (PKC) is a family of serine-threonine kinases known to regulate the epithelial barrier function via tight junctions (Balda et al., 1993; Andreeva et al., 2006). PKC activation can readily disrupt the integrity of pancreatic
Fig. 1. A. Double-immunohistochemical staining for tricellulin (green) and occludin (red) in human nasal mucosa. Nuclear staining: DAPI. B. Immunocyto staining for tricellulin in human nasal epithelial cells in vitro. C. Immuno-transmission electron microscopy (I-TEM) for tricellulin in human nasal epithelium. Arrows: tricellulin-associated gold particles. D. Double-immunohistochemical staining for tricellulin (green) and CD11c (red), LSR (green) and CD11c (red) in human nasal mucosa. Nuclear staining: DAPI. Scale bars: A, D, 40 µm; B, 10 µm; C, 250 nm.
epithelial tight junctions by causing ROCK-II dependent actomyosin-driven contractility or remodeling of the spectrin-adducin based membrane skeleton (Ivanov et al., 2009; Naydenov and Ivanov, 2010).

To investigate how tricellulin is regulated in human pancreatic duct epithelial cells, we used various cytokines, growth factors and signal transduction inhibitors. We found dramatic changes of tricellulin and the barrier function in HPAC cells induced by the c-Jun N-terminal kinase (JNK) activators anisomycin and TPA, and the proinflammatory cytokines IL-1β, TNFα and IL-1α (Fig. 3C) (Kojima et al., 2010). Furthermore, the JNK inhibitor SP600125 and NF-κB inhibitor IMD-0354 prevented tricellulin expression in HPAC cells (Fig. 3C) (Kojima et al., 2010). In normal human pancreatic duct epithelial cells in vitro, tricellulin expression in response to the various stimuli was similar to that in HPAC cells (Kojima et al., 2010).

JNK, known as stress-activated protein kinase, belongs to the mitogen-activated protein kinase (MAPK) group of serine threonine protein kinases and its cascade plays an important role in cell proliferation, differentiation and apoptosis (Davis, 2000; Karin and Gallagher, 2005; Weston and Davis, 2007). JNK signaling is also involved in cell scattering during epithelial to mesenchymal transition in pancreatic epithelial cells (Shintani et al., 2006). JNK activation is essential for disassembly of adherens and tight junctions in human keratinocytes and colonic epithelial cells (Lee et al., 2009; Naydenov et al., 2009). Recently, inhibition of JNK activity was shown to enhance epithelial barrier function through differential modulation of claudin expression in murine mammary epithelial cells (Carrozzino et al., 2009). Furthermore, some claudins are regulated via the NF-κB pathway (Kamekura et al., 2010; Masaki et al., 2011).

Our results suggest that JNK and NF-κB are largely involved in the regulation of tricellular tight junctions, including TRIC expression and the barrier function during normal remodeling of epithelial cells, and prevent disruption of the epithelial barrier in inflammation and other disorders in pancreatic duct epithelial cells.

### Tricellulin in mouse Schwann cells

Autotypic tight junctions are observed as tight junction strands between adjacent cell membranes in the inner and outer mesaxon, paranodal loops, and Schmidt-Lanterman incisures in the peripheral myelin sheath by freeze-fracture electron microscopy (Sandri et al. 1977; Tetzlaff 1978, 1982). They are composed of various transmembrane and peripheral cytoplasmic tight junction proteins, including claudin-19 and junctional adhesion molecule (JAM)-C (Miyamoto et al. 2005; Scheiermann et al. 2007).

We investigated whether tricellulin was expressed in three regions of myelinating Schwann cells: the paranodal loops, Schmidt-Lanterman incisures, and outer/inner mesaxons. The expression level of tricellulin mRNA is about 10-fold higher in the sciatic nerve than...
Tricellulin in epithelial cells and non-epithelial cells

Fig. 3. A. Double-immunocyto staining for tricellulin (green) and occludin (red), LSR (green) and occludin (red) in HPAC cells. Nuclear staining: DAPI. B. Immuno-transmission electron microscopy (I-TEM) for tricellulin in HPAC cells. TC: tricellular contact. Arrows: tricellulin-associated gold particles. C. Western blotting for tricellulin in HPAC cells after treatment with 1 µM anisomycin or 10 µM SP600125. TRIC: tricellulin. Scale bars: A, 10 µm; B, 50 nm.
Tricellulin in epithelial cells and non-epithelial cells

Tricellulin is strongly concentrated at the paranodal loops, Schmidt-Lanterman incisures, and mesaxons of the myelinating Schwann cells (Fig. 4A). There is a gap between tricellulin and Na⁺ channels in the thin region of the paranode indicated by transmission electron microscopy (Fig. 4B,C). Furthermore, tricellulin is in part colocalized with claudin-19 and JAM-C in the three regions of myelinating Schwann cells (Kikuchi et al., 2010, personal data).

It is possible that tricellulin may help to maintain the integrity for function and morphology of peripheral nervous system (PNS) myelin, although the changes in expression and distribution of tricellulin remain unknown under pathophysiological conditions such as peripheral nerve crush injury.

Tricellulin in rat and human hepatocytes

Hepatocytic tight junctions play crucial roles in the barrier to keep bile in bile canaliculi away from the blood circulation, which we call the blood-biliary barrier (Kojima et al., 2003; Kojima and Sawada, 2011). The hepatic tight junction proteins occludin, JAM-A and claudin-1, -2, -3 are expressed in the regions of bile canaliculi and they are also regulated by various cytokines and growth factors via distinct signal transduction pathways (Kojima et al., 2009a,b).

In murine livers, tricellulin is detected together with occludin, JAM-A, CAR and claudin-1, -2, -3, -5, -7, -8, -12, -14 and expressed in the regions of bile canaliculi (Fig. 5) (Kojima et al., 2009a). Furthermore, LSR is also expressed in the regions of bile canaliculi and colocalized with tricellulin (Fig. 5). In human livers, tricellulin is also detected together with occludin, JAM-A, ZO-1, ZO-2 and claudin-1, -2, -3, -7, -8, -12, -14 and can be observed as a pair of spots in the regions of bile canaliculi where tight junctions can be identified as a set of branched intramembranous strands in freeze fracture replicas (Fig. 5) (Kojima et al., 2009a).

Claudin-1 and occludin act as coreceptors of hepatitis C virus (HCV) in the late stage of entry into hepatocytes (Mee et al., 2008; Benedicto et al., 2009). Furthermore, claudin-2 and JAM-A play crucial roles in bile canicular formation (Konopka et al., 2007; Son et al., 2009). In the regions of bile canaliculi of hepatocytes, the distribution and the role of tricellulin may be different from those of ductal epithelial tricellulin.

Conclusion

More recently, it has been reported that sodium caprate, which acts as an absorption enhancer for macromolecules by modulating the paracellular pathway, increases permeability in tricellular contacts together with a marked reduction of tricellulin in intestinal cells (Krug et al., 2013). Shigella targets epithelial tricellular junctions including tricellulin to spread between epithelial cells (Fukumatsu et al., 2012). Tricellulin in epithelial cells may play a crucial role not only in prevention of the passage of various antigens and pathogens but also in drug delivery systems.

On the other hand, in cancer, tricellulin expression is
reduced in hepatic fibrolamellar carcinoma and tonsillar squamous cell carcinoma compared to normal tissues (Kondoh et al., 2011; Patonai et al., 2011). Well-differentiated pancreatic ductal adenocarcinomas significantly overexpress tricellulin as compared with poorly differentiated adenocarcinomas (Korompay et al., 2012). Tricellulin expression in gastric carcinoma cells is negatively regulated by snail-induced epithelial-mesenchymal transition (EMT) (Masuda et al., 2010). Knockdown of LSR increases cell motility and invasion in bladder cancer cells (Herbsleb et al., 2008). Tricellulin is closely related with the degree of cell differentiation, although the changes of LSR are unclear in cancer cells. Tricellular junction proteins may also be a promising molecular target in the diagnosis and therapy for cancer cells like claudins.

At present, the mechanisms of regulation of tricellulin expression of DCs and the interaction between DCs and epithelial cells at tricellular corners remain unknown. However, it is thought that transcellular migration of inflammatory cells in part targets tricellular cell corners. The regulation of tricellular junctions, and tricellulin in epithelial cells and non-epithelial cells, is also important in innate immunity.

Fig. 5. A. Double-immunohistochemical staining for tricellulin (green) and LSR (red) in rat liver. B. Immunohistochemical staining for tricellulin in human liver. Nuclear staining: DAPI. C. Freeze-fracture replica (FF) in human liver. Scale bars: A, 40 µm; B, 10 µm; C, 100 nm.
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Conflicts of interest. The authors declare no conflicts of interest.

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