Pathogenic mechanisms in microvillus inclusion disease
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Chapter 6

LOSS OF INTESTINAL MYOSIN VB FUNCTION IMPAIRS CLAUDIN-1 TRAFFICKING IN MICROVILLUS INCLUSION DISEASE

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(Manuscript to be submitted)
Abstract

Microvillus inclusion disease (MVID) is one of the most severe congenital diarrheal disorder affecting young children and is characterized by MYO5B mutations, impaired brush border development and severe chronic secretory diarrhea. The aim of this study was to investigate the link between the loss of myosin Vb function and intestinal epithelial barrier function. We found that loss of myosin Vb expression or function in the epithelial cells of MVID duodenum and colon and in human intestinal epithelial Caco-2 cells impaired the trafficking of claudin-1 to tight junctions, but did not affect other examined tight junction- or adherens junction-associated proteins. The resultant intracellular retention of claudin-1 was not accompanied by an enhanced paracellular permeability to macromolecules. These data indicate that myosin Vb controls the trafficking of claudin-1, but that an intestinal epithelial barrier defect by means of a macromolecular leak flux mechanism is not likely to account for the chronic diarrhea in MVID patients.

Introduction

Mutations in the MYO5B gene, encoding the recycling endosome associated motor protein myosin Vb, have recently been associated with Microvillus Inclusion Disease (MVID) (Muller et al, 2008; Erickson et al, 2008; Szperl et al, 2011). To date, 41 distinct homozygous and (compound) heterozygous mutations have been identified in forty MVID patients (van der Velde et al, 2013). MVID patients develop nutrient malabsorption and intractable neonatal secretory diarrhea which is aggravated upon oral food intake (Davidson et al, 1978; Philips et al, 1985; Cutz et al, 1989). Patients depend on total parenteral nutrition for survival. At the cellular level, MVID is characterized by microvillus atrophy, and the intracellular retention of brush border proteins (Davidson et al, 1978; Cutz et al, 1989; Philips et al, 1992) including the main brush border anion transporter cystic fibrosis transmembrane conductance regulator and the NHE-2 and -3 Na+/H+ exchangers (Ameen et al, 2007) and some basolateral proteins (Muller et al, 2008; Thoeni et al, 2013). MYO5B mutations have been correlated to, at least in some patients, reduced expression of the myosin Vb protein in the MVID intestine (Szperl et al, 2011) Despite the identification of the affected gene, the underlying disease mechanism, in particular the cause of the chronic diarrhea, remains unclear. So far, small bowel/colon transplantation is the only way to end the secretory diarrhea in MVID.

The intestinal epithelial cells form a tight monolayer along the crypt-villus axis of the intestine, and serve as a permeability barrier between luminal contents and the tissue (Flier et al, 2009). Tight junctions form a physical intercellular and intramembranous barrier at the apex of the lateral (contacting) surfaces in epithelial cell monolayers that control the paracellular transport of ions and water. In addition, tight junctions are tightly associated with apical-basal polarity and prevent the lateral diffusion of membrane proteins between the apical and basolateral plasma membrane domains (reviewed in Giepmans and van IJzendoorn, 2009; Sawada N, 2013; Suzuki T, 2013). When tight junctions of intestinal epithelial cells are disrupted, uncontrolled paracellular leakage of solutes and water occurs, resulting in diarrhea (Suzuki T, 2013; Aiyaz et al, 2006; Laukoetter et al, 2006). Tight junctions consist of transmembrane and associated cytoplasmic proteins.
Transmembrane proteins include occludin, junction-associated membrane protein A, and various members of the claudin family (Chiba et al, 2008). TJ-associated cytoplasmic proteins include Zona Occludens (ZO)-1, -2 and -3, cingulin, and others. The development and maintenance of tight junctions is linked at the molecular level to that of other cell-cell junctions (Ceriejido et al, 2000; Contreras et al, 2002) and Epcam (Ladwein et al, 2005; Lei et al, 2012) although in at least some cell types the formation of functional tight junctions is not strictly dependent on adherens junctions (Theard et al, 2007).

Tight junctions are dynamic structures and some tight junction proteins undergo endocytosis and recycling back to the plasma membrane (Yu and Turner, 2008; Chalmer and Whitney, 2012; Dukes et al, 2011). Interestingly, recycling endosomes have been shown to control the intracellular trafficking of key protein components of tight junctions as well as adherens junctions (Lock et al, 2005; Langewin et al, 2005; Desclozeau et al, 2008; Utech et al, 2010). Whether myosin Vb is involved in the regulation of trafficking of tight junction proteins is not known. We hypothesized that defects in tight junction function caused by loss of myosin Vb function could help explain the chronic diarrhea in MVID patients in the absence of brush border (an) ion transporters and oral food intake. Therefore, we investigated the involvement of myosin Vb, as a key regulator of recycling endosome function (Lapierrre et al, 2001), in the organization and function of tight junctions in intestinal epithelial cells in the context of MVID.

Results and Discussion

Loss of myosin Vb function causes the redistribution of claudin-1 but not of claudin-7, ZO-1 and cingulin in MVID enterocytes

We first determined the subcellular distribution of tight junction proteins (i.e., the sealing claudin-1, the channel-forming claudin-7, cingulin, and ZO-1) in two patients diagnosed with MVID by confocal laser scanning microscopy. Patient 1 carried a homozygous c.4366C>T mutation in the MYO5B gene, and patient 2 carried compound heterozygous c.1540T>C and IVS33+3753G>C mutations in the MYO5B gene (Szperl et al, 2011). Both patients presented persistent secretory diarrhoea from birth on and this disappeared after receiving a bowel transplantation (Golachowska et al, 2012). Immunolabeling was performed on both duodenal and colonic material of both MVID patients.

Claudin-1 localized at the apical domain of epithelial cells in control duodenum (Figure 1A) and colon (supplementary Figure 1) and some intracellular staining was observed. In contrast, in epithelial cells in MVID duodenum (Figure 1A) and colon (supplementary Figure 1) claudin-1 was absent from the apical domain and displayed a pronounced intracellular accumulation close to the nucleus. A difference in the intracellular distribution of claudin-1 was noted between the two patients, as claudin-1 in MVID [c.1540T>C and IVS33+3753G>C] displayed a more condensed supranuclear appearance when compared to MVID [c.4255C>T].

In contrast to the strikingly different distribution pattern of claudin-1 between control and MVID enterocytes, claudin-7, cingulin and ZO-1 predominantly localized at the lateral plasma membrane and/or apical aspect of the lateral plasma membrane domains in
duodenal biopsies (Figure 1A) and colon biopsies (supplementary Figure 1) from both control and MVID patients, consistent with their distribution profiles reported in other studies (Ding et al, 2012). It was noted that a larger fraction of claudin-7 appeared in vesicular structures in the cytoplasm of control duodenum when compared to both MVID samples, while in MVID \([c.1540T>C \text{ and } \text{IVS}33+3753G>C]\) duodenum claudin-7 accumulated more to the apical side of the lateral plasma membrane (Figure 1A).

<table>
<thead>
<tr>
<th>Control</th>
<th>MVID ([c.4366C&gt;T])</th>
<th>MVID ([c.1540T&gt;C + \text{IVS}33+3753G&gt;C])</th>
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<tr>
<td>Claudin-1</td>
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<td>Claudin-7</td>
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<td>Zo1</td>
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To establish a causal relationship between myosin Vb function and claudin-1 distribution, we used human Caco-2 cells, which can be cultured as differentiated and polarized cells such that their phenotype resembles the enterocytes of the small intestine (Hidalgo et al, 1989; Halbleib et al, 2007). The knockdown of myosin Vb in Caco-2 cells using lentiviral

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**Figure 1. Loss of myosin Vb causes redistribution of claudin-1**

A) Sub-cellular distribution of Claudin-1, Claudin-7, Cingulin and Zo1 in Control and enterocytes of two MVID patients with distinct mutations. Solid white arrows show TJ or lateral staining of these proteins. Yellow arrows show intracellular mislocalisation of these proteins. Asterisk indicates the apical lumen in the picture. The dotted line indicates the apical surface in the tissue. Nuclei are stained with DAPI and shown in blue.

B) Sub-cellular distribution of claudin-7, cingulin, Zo1 and claudin-1 in Caco2 cells is depicted in the x-z axis as side views in red. The cells transduced with lentiviral virus express GFP shown in green while non-transduced cells are controls and do not show GFP. White arrows indicate TJ or lateral junction and normal distribution. Yellow arrows indicate intracellular mislocalisation of these proteins due to the knockdown. Nuclei are stained blue.
transduction of shRNA against myosin Vb, which has been shown to reproduce phenotypic hallmarks of MVID enterocytes (Ruemelle et al, 2010), replicated our observations in the MVID intestine (Figure 1B). Thus, no overt differences were observed in the subcellular distribution of claudin-7, cingulin and ZO-1 in GFP-negative (uninfected/control) and GFP-positive (infected) Caco-2 cells. (Figure 1B, side view/x-z images, white arrows). In contrast, claudin-1 was clearly redistributed from the lateral surface in control cells to the intracellular, supranuclear region in myosin Vb knockdown cells (Figure 1B).

Together, these data demonstrate that loss of myosin Vb function can be causally linked to the redistribution of claudin-1 in MVID enterocytes.

Loss of myosin Vb function impairs trafficking of claudin-1 to tight junctions

The intracellular accumulation of claudin-1 in myosin Vb knockdown Caco-2 cells and MVID enterocytes suggested that myosin Vb regulated the trafficking of claudin-1 to the tight junctions. In order to investigate this further, we used an experimental approach in which endocytosis of tight junction proteins is first triggered in a confluent monolayer of cells by treatment with the Ca²⁺-chelator ethylenediaminetetraacetic acid (EDTA) (Ivanov et al, 2004) and after subsequent washout of the EDTA, the trafficking of these proteins is followed while they form new tight junctions as function of time. Treatment of control and myosin Vb knockdown Caco-2 cell monolayers with EDTA resulted in the redistribution of ZO-1 and of claudin-1 from the apex of the lateral plasma membrane to vesicular structures in the apical cytosol (Figure 2). In EDTA-treated myosin Vb knockdown cells, intracellular claudin-1 displayed a more condensed accumulation in the apical cytoplasm and also residual plasma membrane associated claudin-1 disappeared (Figure 2), suggesting that myosin Vb was not required for the endocytosis of claudin-1. After 120 min of EDTA washout, ZO-1 reappeared at the apex of the lateral plasma membrane, indistinguishable from the situation before EDTA treatment, in both control and myosin Vb knockdown cells (Figure 2). At this time, a fraction of claudin-1 had also reappeared at the lateral surface of control cells although a significant fraction of intracellular claudin-1 was still observed (Figure 2). In myosin Vb knockdown cells, in contrast, claudin-1 was mainly intracellular and no claudin-1 had appeared at the cell surface (Figure 2). After 240 min in control cells, claudin-1 localized at the lateral surface indistinguishable from the situation before EDTA treatment, whereas in myosin Vb knockdown cells claudin-1 remained intracellular (Figure 2). Together these data demonstrate that myosin Vb regulates the trafficking of claudin-1 to the tight junctions, and that claudin-1 at tight junctions is dispensable for the recruitment of ZO-1 and cingulin at tight junctions in human enterocytes.

Loss of myosin Vb function does not affect the lateral distribution of beta-catenin and EpCam or the monolayer organization of intestinal epithelial cells

The establishment and maintenance of tight junctions is linked to that of adherens junctions (Ladwein et al, 2005; Lei et al, 2012). We found that the key adherens junction-associated
protein beta-catenin displayed a normal distribution along the lateral plasma membrane in MVID enterocytes and/or shRNA-treated Caco-2 cells (Figure 3A). Also the lateral plasma membrane localization of the cell-cell adhesion protein EpCam mutated forms of which fail to reach the plasma membrane and cause congenital tufting enteropathy (Sivagnanam et al, 2008; Schnell et al, 2013; Goulet et al, 2007) was maintained in shRNA-treated Caco-2 cells (Supplementary Figure 2; the EpCam antibody did not work properly on our paraffin coupes). It was noted that in control Caco-2 cells EpCam in addition localized to the apical surface, and this apical (but not the lateral) fraction of EpCam was depleted in myosin Vb knockdown cells (supplementary Figure 2). In agreement with the localization of most cell-cell adhesion proteins at the lateral surface, the intestinal epithelial cell monolayer

Figure 2) Myosin Vb impairs trafficking of claudin-1. White arrows indicate normal localization of claudin-1 at the cell periphery. Yellow arrows indicate abnormal intracellular accumulation of Claudin-1. A) Localization of claudin-1 (red) and Zo1 (blue) in confluent monolayer of Caco2 cells which are un-infected lentivirus for Myosin Vb knockdown. B) Localization Claudin-1 in cells transduced with lentiviral for Myosin Vb knockdown and expressing GFP (green). C-D) Cells treated with calcium chelator EDTA and then allowed to recover for time points 5 min to 240 min post EDTA treatment and stained for Claudin-1 (red). Non-green cells are control cells without viral transduction while green cells are lentivirus transduced cells with GFP (green). Yellow arrows indicate the enlarged intracellular accumulation while white arrows indicate normal distribution of Claudin-1 and Zo-1.
Figure 3) Loss of myosin Vb does not affect adhesion, monolayer organization and permeability. 

A) Beta-catenin (green) localization in control and MVID patient enterocytes. Nuclei stained with DAPI (blue). Asterisk indicates the apical lumen and dotted line is the apical plasma membrane. 

B) MVID patient intestinal tissue stained with giemsa indicates the organisation of the tissue and enterocyte monolayer. 

C) Transmission electron micrographs indicate microvillus inclusion (MI) in MVID enterocyte. Black dotted box is blown up on right. Black arrows indicate the electron dense tight junctions. Blue arrows indicate cell-cell adhesions and red arrows indicate desmosomal junctions. 

D) Cartoon depicting the arrangement for the flux assay to establish paracellular permeability coefficient in confluent Caco2 cells. Green is FITC-Dextran 4kda, red show tight junctions. Cells are grown on porous polycarbonate filter. Bottom chamber is the receiving chamber for diffusing FD4 gradient. Apparent coefficient is calculated by the given formula. 

E) Papp plotted for control uninfected cells, knockdown cells, EDTA treated cells and empty filter. Student’s t-test was performed between two samples. p<0.05 was considered significant. The graph was plotted as an inverted plot with Y-axis on log scale to the base 10.
arrangement in MVID, despite the fusion of villi, appeared normal by hematoxylin and eosin staining (Figure 3B), and also Caco-2 cells that were treated with shRNA against myosin Vb displayed a normal monolayer arrangement (Figure 1B and 2). Moreover, cell-cell junctions in MVID intestinal epithelial cells displayed a normal morphology when evaluated by transmission electron microscopy (Figure 3C). Indeed, typical electron-dense desmosomes (blue arrows) and tight junctions seen as characteristic electron-dense areas of closely opposing membranes between cells were easily distinguishable at the apex of the lateral membrane (double black arrow, Figure 3C; red arrows indicate areas with visible space between the opposing lateral plasma membranes).

**Loss of myosin Vb function does not increase paracellular permeability to macromolecules**

Because loss of myosin Vb function in vivo and in vitro led to the redistribution of claudin-1, we next addressed the consequences of myosin Vb loss of function for the epithelial barrier function. For this, control and myosin Vb knockdown Caco-2 cells were cultured as confluent monolayers on semi-permeable filters. Fluorescein isothiocyanate -labelled 4 kDa dextran (FD4) was added to the apical side of the monolayer and the FD4 that leaked to the basolateral compartment was calculated as a function of time (Figure 3D). As a positive control, cells were treated with EDTA to disrupt the tight junctions (Figure 2). Whereas treatment with EDTA caused a significant increase in paracellular leakage of the FD4 (Figure 3E), no significant difference in FD4 leakage was observed between control and myosin Vb knockdown cells (Figure 3E). These data demonstrated that in Caco-2 cells, the reduced expression of myosin Vb and resultant redistribution of claudin-1 did not lead to an impaired tight junction and barrier integrity. In agreement with this, also the knockdown of claudin-1 itself in Madin-Darby canine kidney epithelial cells did not result in increased paracellular leakage (Goulet et al, 2007).

Our data indicate that although loss of myosin Vb expression or function causes impaired trafficking and intracellular retention of the sealing tight junctional protein claudin-1, it is not paralleled by an increase in the paracellular leakage of macromolecules. This contrasts an earlier reported increase in paracellular transport of the 4.4 kDa horseradish peroxidase in duodenal biopsies of MVID patients (Bijlsma et al, 2000). The reason for this inconsistency is not clear. MYO5B mutations have been confirmed in the patients in our study, whereas the MYO5B genes in the patients in the Bijlsma study have not been analysed. As at least one MVID patient without MYO5B mutations has been reported (Muller et al, 2008), it is possible that the biopsies used in the Bijlsma study were from patients with a different genetic cause and, therefore, pathogenesis. Furthermore, in support of our findings, MVID is not associated with intestinal inflammation or immune cell infiltration, which would be expected if the intestinal epithelial barrier function was compromised. In light of the redistribution of tight junction proteins including claudin-1 that accompanies intestinal inflammatory conditions (Ivanov et al, 2004), our data also argues against a causative role of claudin-1 redistribution in intestinal inflammation. Finally, the
presence of functional tight junctions in MVID intestinal epithelial cells also explain why apical and basolateral membrane proteins do not intermix in the plasma membrane of MVID enterocytes, and underscores that MVID is caused by impairment of plasma membrane-directed trafficking and not due to defects in the epithelial cell polarity program as such.

All together, we conclude that - despite the impaired trafficking and redistribution of claudin-1 - cell-cell adhesion and tight junction function is maintained upon loss of myosin Vb function. Consequently, a substantial intestinal epithelial barrier defect by means of a macromolecular leak flux mechanism is not likely to account for the chronic diarrhoea in MVID patients.

**Methods**

**Cell culture**

Caco-2 cells were cultured in DMEM with non-essential amino acids (Sigma) and 10% foetal calf serum (FCS) as described in earlier chapters.

**Knockdown in CaCo2 cells**

The shRNA target sequence, region 2333-2351 of human MYO5B cDNA (target sequence GGCTGCAGAAGGTGAAATA) was cloned into the shRNA expression vector described previously. A target sequence in the Luciferase gene was used as a control. For the production of lentivirus HEK293T cells were plated onto poly-L-Lysine-coated plates in DMEM supplemented with 10% FCS and 1% sodium pyruvate. Cells were transfected with CMVdR8.1, VSV-G, and pMID-i-2 using CaCl2 and Hank’s balanced salt solution. Medium was changed after 17 h. After 24 h virus-containing medium was collected, filtered and stored at -80°C. Caco-2 cells were transduced with lentivirus diluted in DMEM with 10% FCS. Expression of GFP in cells was indicative for successful transduction, as also evidenced by reduced myosin Vb mRNA. In other experiments, Caco-2 cells/Transwell filter (Corning, 0.4 micron pore size) were plated and 48 h later transduced with virus in the presence of polybrene (1:1000) for 16 h, and cultured for another 4 days.

**Calcium switch assay**

CaCo2 cells were plated on transwell filters and transduced with lentivirus on day 2 post seeding. The cells were allowed to grow for six days in total. The cells were washed with phosphate buffered saline (PBS) without calcium and magnesium thrice before adding 2.5mM EDTA in PBS. The cells were incubated in EDTA for 8 mins, washed twice with PBS and later fixed with 4% PFA in PBS at various time points.

**Immunofluorescence labeling and microscopy**

Sections of formalin-fixed samples of MVID and control intestines were de-paraffinised, rehydrated, washed with PBS and subjected to epitope retrieval with citric acid pH 6.0 in a microwave for 20 min. Non-specific binding sites were blocked with 5% FCS in PBS
overnight. Primary antibodies were diluted in blocking solution with 0.05% Tween-20 at 37°C for 2h followed by incubation with AlexaFluor-488- or -543-conjugated secondary antibodies. Nuclei were stained with DRAQ5, and slides were mounted with DAKO mounting medium. Cultured cells were fixed with 3.7% PFA at room temperature for 20 min. Cells were incubated with 0.1 M glycine in PBS for 20 min, permeabilized with 0.2% TritonX-100 for 10 min and blocked with 3% FCS in PBS for 1 h. 30 and primary antibodies were added to the basolateral and apical side of the filter and cells were incubated at 37°C for 2 h. Cells were incubated with Cy5- or AlexaFluor-543-conjugated secondary antibodies and DRAQ5/DAPI at 37°C for 30 min. Filters were mounted in DAKO mounting medium. Specimens were examined and images were taken with a TCS SP8 CLSM (Leica). Claudin-1 (1:100 Invitrogen), claudin-7 (1:100 Sigma), cingulin (1:100 Novus Biologicals), zo-1 (Invitrogen 1:200), beta-catenin (1:100 Transduction lab).

**FD4 paracellular leakage assay**

CaCo2 cells were plated on transwels, transduced with the virus and allowed to grow to form a monolayer as mentioned above. On day 6, all transwels were washed with PBS twice. Add 1.5ml PBS (or 2.5 mM EDTA in PBS as control) on the basal side and 250 µg/ml FD4 in PBS (or 250 µg/ml PBS + 2.5mM EDTA as control). The cells were incubated with FD4 for 20min and the basal medium was collected in a tube and replaced with fresh PBS (or PBS with EDTA). Subsequent samples were collected every 20 mins for 120 mins. As a blank, an empty transwell filter was used to calculate the basal rate of flux between the apical and basal chambers. The sequentially collected basal solutions were read on fluorimeter with a standard curve of fluorescence intensity to FD4 concentration. The apparent permeability coefficient ($P_{app}$) was calculated using the following formula: $P_{app} (cm/s) = \frac{dQ/dt \times 1/A \times Co}{dQ/dt (\mu g/s)}$ where $dQ/dt (\mu g/s)$ is the rate of appearance of FD4 on the receiver side from 20 to 120 min after application of FD4. $Co (\mu g/m)$ is the initial FD4 concentration on the donor side, and $A (cm2)$ is the effective surface area of the insert.
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**Supplementary information**

Supplementary figure 1. Loss of myosin Vb causes redistribution of claudin-1. Sub-cellular distribution of Claudin-1, Claudin-7, Cingulin and Zo1 in biopsies of colon in two MVID patient samples with distinct mutations and a control sample. Solid white arrows show intracellular accumulation of claudin-1 in MVID colon staining. Asterisk indicates the apical lumen in the picture. The dotter line indicates the apical surface in the tissue. Nuclei are stained with DAPI (blue).

Supplementary Figure 2. Loss of myosin Vb in Caco2 cells does not affect EpCAM distribution in polarized Caco2 cells. Subcellular distribution of EpCAM (red) in MYOVB ShRNA transduced (green) and untransduced Caco2 cells. (scale bar 10um)