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Immunohistological characterization of intercellular junction proteins in rhesus macaque intestine

Sanjeev Gumber, DVM, PhD, DACVP^{1,3,*}, Asma Nusrat, MD^{3,4}, and Francois Villinger, DVM, PhD^{2,3}

¹Division of Pathology, Yerkes National Primate Research Center, Atlanta, Georgia, USA

²Division of Microbiology and Immunology, Yerkes National Primate Research Center, Atlanta, Georgia, USA

³Department of Pathology and Laboratory Medicine, Emory University School of Medicine, Atlanta, Georgia, USA

⁴Department of Cell Biology, Emory University School of Medicine, Atlanta, Georgia, USA

Abstract

Epithelial junctions play an important role in regulating paracellular permeability and intercellular adhesion. It has been reported that changes in the density of epithelial junctions and/or distribution pattern can contribute to various gastrointestinal (GI) disorders. In this study, we investigated the distribution of the tight junction (Claudins 1, 3, 4, 5, 7, 10, Zonula Occludens (ZO-1), Occludin), adherens junction (E-cadherin), desmosome (Desmoglein 2, Desmocollin 2) and gap junction (Connexin 43) proteins in the jejunum, ileum and colonic epithelium of healthy rhesus macaques (RM) using immunofluorescence labeling. While proteins in these respective junctions were expressed throughout the jejunum, ileum and colon of RM, we observed differential labeling in epithelial cells from these sites. Claudins 1, 3, 4, 7, E-cadherin and Desmoglein 2 were distributed in the respective intercellular junctions with additional labeling in the lateral membrane of epithelial cells in both small and large intestine. However, claudin 5, claudin 10, ZO-1 and occludin showed uniform distribution in the intercellular junctions of crypt and surface epithelial cells of the intestine. Desmocollin 2 localized predominantly in the upper two thirds along the lateral membrane while desmoglein 2 was distributed along the entire lateral membrane of intestinal epithelial cells. In contrast, connexin 43 exhibited punctate lateral labeling in crypt epithelial cells of the small and large intestine. Our results show diverse localization of epithelial intercellular junction proteins along the intestinal tract of RM. These findings may correlate with differences in paracellular permeability and adhesion along the intestinal tract and could correlate with pathologic disease in these regions of the intestine.

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*Corresponding author: Sanjeev Gumber, Division of Pathology, Yerkes National Primate Research Center, Emory University, 954 Gatewood Road NE, Atlanta, Georgia 30329, USA. Tel.: +1 404 727 7022 Fax: +1 404 727 4531 sgumber@emory.edu.

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Keywords

Claudins; Connexins; Desmosome; Epithelial junctions; Immunohistochemistry

1. Introduction

A simple columnar intestinal epithelium functions as an important barrier that separates luminal contents from underlying tissues (Groschwitz and Hogan, 2009, Koch and Nusrat, 2012). Epithelial barrier properties are achieved by the formation of complex protein-protein networks that mechanically link adjacent cells and seal the intercellular space. These protein networks connecting epithelial cells form intercellular junctions that include the adherens junction (AJ), tight junction (TJ) and desmosomes (DM) (Groschwitz and Hogan, 2009). TJ regulate paracellular permeability across epithelial cells while AJ and DM serve as adhesive contact that maintains mechanical integrity of the epithelium barrier (Capaldo et al. , 2014, Turner, 2009). The TJ and AJ associate with underlying filamentous actin (F-actin) and desmosomes provide structural strength to epithelium through their association with the intermediate filaments (Guttman et al. , 2007). Among cell-cell adhesion molecules, intercellular communication mediated by gap junction is required for maintaining cellular homeostasis and function (Vinken et al. , 2006). Gap junctions directly link the cytoplasm of neighboring cells and provide a pathway for intercellular exchange of small and hydrophobic substances including ATP and ions (Yeager and Harris, 2007). A gap junction which allows transfer of ions and fluids across the cell membrane is composed of two hemichannels and six transmembrane proteins known as connexins which form one hemichannel (Stanfield and Germann, 2009). Connexins are considered to play an important role in the differentiation of epithelial cells and are associated with AJ and TJ.

There is growing evidence that increases in intestinal permeability play pathogenic roles in diseases (Camilleri et al. , 2012). The significance of TJ in the diseases caused by various pathogens has been documented including *Vibrio cholera* (Wu et al. , 2000), enteropathogenic *Escherichia coli* (Muza-Moons et al. , 2004), *Clostridium perfringens* (Fujita et al. , 2000), and immune mediated diseases such as Inflammatory Bowel Disease (IBD) (Gitter et al. , 2000), and celiac disease (Schulzke et al. , 1998). These diseases are accompanied by alteration of intestinal barrier functions that is linked to compromised TJs. In IBD, increased paracellular permeability contributes to diarrhea due to defective TJ (Ivanov et al. , 2010, Turner, 2006, 2009). A number of studies have documented intestinal barrier dysfunction particularly during chronic stages of the Simian and Human Immunodeficiency Virus (SIV/HIV) infection (Epple et al. , 2009, Estes et al. , 2010, Keating et al. , 1995, Sharpstone et al. , 1999) but the underlying pathophysiological mechanisms are still unclear. During SIV/HIV infection the translocation of microbial products from the gastrointestinal (GI) tract to portal and systemic circulation has been proposed as a causal link to chronic immune activation associated with the disease progression (Marchetti et al. , 2013). Microbial translocation results from a series of immunopathological events in the GI mucosa including early and severe CD4 T-cell depletion; mucosal immune hyperactivation; damage to intestinal epithelial barrier with enterocyte apoptosis and TJ disruption and subverted gut microbiome (Marchetti, Tincati,

2013). In addition, there has been renewed interest in the role of intestinal permeability and TJ in the pathogenesis of chemotherapy-induced gut toxicity (CIGT) (Wardill et al. , 2012). Several studies have documented changes in TJ following chemotherapy administration suggesting a possible role of TJ in the pathophysiology of CIGT (Beutheu Youmba et al. , 2012, Blijlevens et al. , 2005, Hamada et al. , 2010).

Rhesus macaques (RM) are considered excellent models to study human aging and diseases due to their genomic, physiological and immunological similarities to humans (Roth et al. , 2004). RM have been successfully used to study the role of GI tract in HIV pathogenesis (Lackner et al. , 2009). However, comprehensive intestinal epithelial junction studies have not been performed in RM. Therefore, the aim of this study was to examine expression and distribution of the tight junction (Claudins 1, 3, 4, 5, 7, 10, Zonula Occludens (ZO-1), Occludin), adherens (E-cadherin), desmosomal [Desmoglein 2 (Dsg2), Desmocollin 2 (Dsc2)] and gap junction (Connexin 43) proteins in the jejunum, ileum and colon of healthy RM to provide baseline data for further experiments using this animal model.

2. Materials and methods

2.1 Animals

Intestinal tissue samples were collected from eight SIV uninfected Indian rhesus macaques (*Macaca mulatta*) which were primarily assigned as unvaccinated controls in a research trial. The animals were considered healthy based on normal physical examination, laboratory analyses and absence of gross and histological lesions. The age of the animals ranged from 3 (n=4) to 20 (n=4) years. All animals were maintained at the Yerkes National Primate Research Center of Emory University in accordance with the regulations of the Guide from the Committee on the Care and Use of Laboratory Animal Resources. The experiments were approved by the Institutional Animal Care and Use Committee of Emory University, Atlanta, GA, USA and biosafety review boards.

2.2 Selection of antibodies and assay optimization

The antibodies in this study were selected based on available literature in nonhuman primates or human diseases. Before pursuing immunofluorescence staining, the assay was optimized using a range of dilutions for each antibody and different antigen retrieval methods using immunohistochemistry. Table 1 describes the optimal combination of antigen retrieval buffers and antibody clones and dilutions that provided strongest labeling with the least background. Positive controls included samples of kidney, lung, liver and brain from SIV uninfected RM and negative controls with omission of primary antibody.

2.3 Immunofluorescence staining

Jejunum, ileum and colon samples collected during necropsy examination were fixed overnight in 4% paraformaldehyde, embedded in paraffin and sectioned at 4 μ m. The paraffin-embedded sections were subjected to deparaffinization in xylene, rehydration in graded series of ethanol, and rinsing with distilled water. Antigen retrieval was performed by immersing the slides either in a target retrieval solution (Dako, Carpinteria, CA, USA) or 1mM EDTA buffer pH 8.0 at 120°C for 4 minutes in a steam pressure decloaking chamber

(Biocare Medical). The slides were then allowed to cool for 20 minutes, washed with distilled water, and placed in phosphate buffered saline containing 0.2% fish skin gelatin (Sigma) (PBS-FSG) for 5 min. Sections were then blocked with 10% normal goat serum (NGS) diluted in PBS-FSG in a humidified chamber at room temperature for 50 minutes. The primary antibodies to claudins (1, 3, 4, 5, 7, 10), ZO-1, Occludin, E-cadherin, Dsg2, Dsc 2 and Connexin (Cx) 43 were diluted in NGS as described in Table 1 and incubated overnight in a humidified chamber at 4°C. Following incubation the slides were washed twice with PBS containing 0.2% fish-skin gelatin and 0.1% Triton X-100 (PBSFSG-Tx100) for ten minutes each and followed with PBS-FSG. Epithelial junctions' immunofluorescence was revealed using goat anti-mouse or goat anti rabbit secondary antibodies coupled with DyLite® 650 (Abcam, USA). The secondary antibodies were diluted in 10% NGS and incubated in a humidified chamber for 1h at room temperature protected from light. Following incubation, the slides were washed twice with PBS-FSG-Tx100 for ten minutes. Upon completion of immunofluorescence staining, the sections were mounted with ProLong® Gold antifade reagent with DAPI (4',6-diamidino-2-phenylindole) (Life Technologies) as a nuclear counterstain and coverslipped.

2.4 Image acquisition and processing

Confocal microscopy was performed using an LSM 700 confocal microscope (Zeiss) with a Plan-Apochromat primary objective (20×; NA, 1.4). All images were captured using multitrack scanning for each fluorophore to prevent bleed-through, and the pinholes were set to an Airy unit of 1 (equal in size to an Airy disk). Jejunum and ileum images were captured using tile scan function and stitched by Zen software; colonic Z-dimension stacks were taken in 0.5-μm increments; the 405-nm laser was used for the DAPI, the 648-nm laser was used for epithelial junction proteins immunostaining.

3. Results

3.1 Distribution of tight junction proteins

Most claudins were expressed in a relatively uniform fashion in the entire RM intestinal tract but differences in their localization relative to TJ and lateral membrane were noted. Claudins 1, 3, 4, 7 were detected in TJ and subjunctional lateral membrane of colonic and villar surfaces and crypt enterocytes with no obvious differences in the expression levels (Figures 1, 2). Claudin 7 expression appeared to be more in the junction and lateral membrane of luminal epithelial cells vs. crypt epithelium i.e. gradient (Figure 2). In contrast, claudins 5 and 10 showed uniform expression exclusively in TJs of crypt vs. surface epithelial cells with no lateral membrane staining. In addition, claudin 5 expression was also detected in intercellular junctions of endothelial cells of mucosal and submucosal blood vessels (Figure 1). Other TJ proteins including ZO-1 and occludin were localized to the apical region of the lateral membrane consistent with their distribution in TJs of crypt and surface intestinal epithelial cells (Figure 2).

3.2 Distribution of adherens junction proteins

E-cadherin was localized in the lateral membranes of crypt and surface intestinal epithelial cells (Figure 3). No variation in expression level was noticed in the small and large intestine.

3.3 Distribution of desmosomal junction proteins

Dsc2 was localized a spot welds along the lateral membrane of crypt and surface epithelial cells. Most of the DSc2 was distributed in the upper two thirds of the lateral membrane (Figure 3). In contrast, Dsg2 was distributed in spot welds along the entire lateral membrane of crypt and surface epithelial cells (Figure 3). Dsg2 was more abundant than Dsc2 in both small and large intestine.

3.4 Distribution of gap junction proteins

Cx43 was localized exclusively in crypt epithelial cells of small and large intestine. Cx43 was identified as punctate spots between crypt epithelial cells of the colon, jejunum and ileum. Jejunum and ileum appeared to show higher expression of Cx43 than colonic epithelial cells (Figure 3). We did not observe labeling of Cx43 in luminal intestinal epithelial cells.

Antigen retrieval with EDTA buffer yielded strongest signals for the detection of claudins 5, 10, ZO-1 and occludin, whereas faint labelling was detected using citrate based buffer. In addition, there were no obvious differences in the distribution and expression levels of TJ, AJ, DM and GJ proteins in young (3-4 year) vs. older (19-20 year) animals.

4. Discussion

Tight junctions are multi-protein complexes composed of integral transmembrane proteins and associated cytoplasmic proteins. The integral membrane proteins interact between neighboring cells and fulfill the gate and fence functions of TJ. The cytoplasmic proteins connect TJ to the actin cytoskeleton and that in turn regulates barrier function (Gunzel and Fromm, 2012). Key barrier forming TJ proteins include the tetraspan family of claudin proteins (Tsukita and Furuse, 2000, Tsukita et al. , 2001). Claudin proteins regulate paracellular permeability by forming channels between epithelial cells (Camilleri, Madsen, 2012, Gunzel and Fromm, 2012). Other transmembrane proteins in TJs include the MARVEL proteins that include occludin. Peripheral membrane proteins including ZO-1, ZO-2 and ZO-3 play a crucial role in the maintenance of TJ assembly (Fanning and Anderson, 2009, Rodgers et al. , 2013). Many pathophysiological events including inflammation, interaction with pathogens and neoplasia cause alteration in TJ protein composition. In addition, many pathogens use TJ proteins as receptors to overcome epithelial barriers and enter target cells. Thus, claudins 1, 6, 9 and occludin mediate entry of hepatitis C virus (Benedicto et al. , 2009, Meertens et al. , 2008), claudin 1 is required for dengue virus entry into host cells (Gao et al. , 2010) and claudins 3, 4 are receptors for the enterotoxin of *Clostridium perfringens* (Sonoda et al. , 1999). The expression of claudins was relatively uniform in jejunum, ileum and colon of RM in this study but differed slightly from human, canine, rat and mouse intestines (Fujita et al. , 2006, Lameris et al. , 2013, Markov et al. , 2010, Ohta et al. , 2011). In humans, mRNA quantification revealed that claudins 3, 4, 7 and 8 were predominately expressed in distal parts of the GI tract (Lameris, Huybers, 2013). ZO-1 and occludin staining was localized to apical region of the lateral membrane of colonic epithelial cells in humans with a typical TJ pattern similar to the one reported here in RM (Bertiaux-Vandaele et al. , 2011). Claudin 5 expression was seen in RM

intestine in endothelial cells, similar to its expression in the human intestine (Amasheh et al. , 2005), suggesting that the general expression patterns of TJ proteins were comparable between man and RM. In contrast, similar analysis from dogs and rodents showed different patterns: canine colonic mucosa lacked labeling for claudins 1, 4, 8 and exhibited weak staining for claudin 5 (Ohta, Yamaguchi, 2011). The rat colon revealed expression of claudins 1, 2, 3, 4, 5, 7, 8 and 12 but strongest signals were detected for claudins 1, 3, 4, 5 and 8 (Markov, Veshnyakova, 2010). Claudins 2, 3, 4 and 5 showed uniform expression in the small intestine of rats (Rahner et al. , 2001). The mouse intestine showed expression of claudins 7, 8, 12, 13 and 15 in jejunum, ileum and colon, whereas claudins 6, 9, 10, 11, 14, 16, 18 and 19 were not observed (Fujita, Chiba, 2006). The possible explanation for such species specific variations in claudin expression could reflect species specific differences, tissue specific variations, and/or environmental differences.

The membranous localization of E-cadherin in the intestine of RM was in agreement with other studies in human, canine, rat and mouse intestines (Dogan et al. , 1995, Larsson, 2006, Ohta, Yamaguchi, 2011). AJs consist of cadherins, a family of transmembrane proteins that form strong homotypic interactions with molecules on adjacent cells. E-cadherin interacts with β -catenin and catenin δ 1 which leads to binding of β -catenin to α -catenin (Halbleib and Nelson, 2006, Turner, 2009). Expression of AJs can also be affected by infection and inflammation. HIV-1 infection has been reported to cause substantial redistribution of E-cadherin in the colonic lamina propria compared to normal human colon samples (Streeck et al. , 2011).

Desmosomes are important components of intercellular junction and are composed of transmembrane desmosomal cadherin proteins including Dsg1-4 and Dsc1-3. They provide mechanical strength to epithelial tissues by forming stable cell-cell contacts which are anchored to keratin intermediate filaments (Getsios et al. , 2004). Desmosomal cadherins differ from classical cadherins (E-cadherin and N-cadherin) by their lack of interaction with β -catenin (Wahl et al. , 2000). The human intestinal epithelium show expression of only Dsc2 and Dsg2 (Holthofer et al. , 2007). In this study, Dsg2 was uniformly expressed at the intercellular junctions and subjunctional lateral membranes of colonic, villar surfaces, and crypt enterocytes. Similar expression level has been reported in an earlier study in humans and RM from the colonic samples (Zhang et al. , 2011).

Cx43 is the most predominant and well-studied of the Cxs due to its widespread expression in many cell types including fibroblasts, epithelial cells, hematopoietic cells, neurons, and cardiac neuron crest cells (Laird, 2006, Oyamada et al. , 2005). Punctate staining pattern of Cx43 in the crypts has been reported previously in human colonic samples similar to findings presented here for RMs (Kanczuga-Koda et al. , 2004). However, the authors also identified focal cytoplasmic, paranuclear immunostaining in the mucosal epithelial cells and a strong immunoreactivity in the muscularis mucosae (Kanczuga-Koda, Sulkowski, 2004). Such labeling was not observed in the monkey intestine. Similarly, another study demonstrated no immunoreactivity to Cx43 in the entire circular muscle layer of the human colon (Mikkelsen et al. , 1993). Connexins polypeptides can be detected in the endoplasmic reticulum and Golgi apparatus, the sites for their synthesis, maturation and posttranslational processing (Dbouk et al. , 2009, Falk, 2000). Cytoplasmic accumulation of connexins at the

luminal surface of colonic epithelium has been reported likely due to accumulation of degraded gap junction proteins during replacement of epithelial cells although the pathophysiology is poorly understood (Kanczuga-Koda, Sulkowski, 2004). We speculate that higher staining aggregations in the jejunum and ileum in the present report could be secondary to the high epithelial turnover rate in the small intestine relative to the colon. It is well known that the epithelial lining of the small intestine is in continuous state of replacement and is probably the organ with the fastest turnover (Creamer, 1967). Recently, it has been demonstrated that Cx43 contribute to the diarrhea caused by *Citrobacter rodentium* in mice (Guttman et al. , 2010). A more recent *in vitro* study showed that loss of functional Cx26 expression provided protection against GI bacterial pathogens, and Cx26 represents a potential therapeutic target for GI bacterial infection (Simpson et al. , 2013). Therefore, accumulating evidences suggest the emerging roles of connexins in GI pathophysiology. Connexins are also considered to be associated with or regulate the expression and function of AJ and TJ (Kojima et al. , 2007, Nagasawa et al. , 2006).

Taken together, the results from this study demonstrate that the profile of epithelial junction expression in RM GI is very similar to the human one, highlighting the validity of RM as a suitable experimental model to study the functions of epithelial junctions.

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Figure 1.

Expression of epithelial junction proteins in jejunum, ileum and colon of rhesus macaques: Claudin 1 (A, B, C), Claudin 3 (D, E, F), Claudin 4 (G, H, I) and Claudin 5 (J, K, L) in jejunum, ileum and colon of rhesus macaques visualized by immunofluorescence (DyLight® 650-labeled antibodies appear green; DAPI-stained nuclei appear blue, 200×). Claudins 1, 3, 4 were detected in intercellular junctions and lateral membrane of colonic and villar surfaces and crypt enterocytes. Claudin 5 showed expression in intercellular junctions of crypts and surface epithelial cells in the small and large intestine. Arrows indicate positive claudin 5 staining in the intercellular junctions of endothelial cells of mucosal and submucosal blood vessels.

Figure 2.

Expression of epithelial junction proteins in jejunum, ileum and colon of rhesus macaques: Claudin 7 (A, B, C), Claudin 10 (D, E, F), Zonula Occludens-1 (G, H, I) and Occludin (J, K, L) in jejunum, ileum and colon of rhesus macaques visualized by immunofluorescence (DyLight® 650-labeled antibodies appear green; DAPI-stained nuclei appear blue, 200×). Claudin 7 was detected in intercellular junctions and lateral membrane of colonic and villar surfaces and crypt enterocytes. Claudin 10 was detected in intercellular junctions of crypts and surface epithelial cells with no lateral membrane staining. ZO-1 and occludin were localized to intestinal crypts and apical membranes of villi and surface epithelium of the colon.

Figure 3.

Expression of epithelial junction proteins in jejunum, ileum and colon of rhesus macaques: E-cadherin (A, B, C), Desmocollin 2 (D, E, F), Desmoglein 2 (G, H, I) and Connexin 43 (J, K, L) in jejunum, ileum and colon of rhesus macaques visualized by immunofluorescence (DyLight® 650-labeled antibodies appear green; DAPI-stained nuclei appear blue, 200×). E-cadherin and Desmoglein 2 were localized in the lateral membranes of crypt and surface intestinal epithelial cells of small and large intestine. Desmocollin2 was expressed in the upper two thirds of the lateral membrane of mucosal surface and crypt epithelium of small and large intestine. CX43 showed punctate staining spots between crypt epithelial cells of the colon, jejunum and ileum.

Table 1
Details of primary antibodies and antigen retrieval methods used in immunofluorescence analysis

Epithelial junction antibody	Antibody type	Clone/catalogue number	Antigen Retrieval Pretreatment	Antibody Dilution	Manufacturer
Claudin-1	Rabbit polyclonal	Ab15098	Target Retrieval Solution (TRS), Citrate, Dako	1:500	Abcam
Claudin-3	Rabbit polyclonal	PAD: Z23.JM, 34-1700	TRS, Dako	1:500	Life Technologies
Claudin-4	Rabbit polyclonal	Ab53156	TRS, Dako	1:1500	Abcam
Claudin-5	Rabbit polyclonal	PAD:Z43.JK, 34-1600	1mM EDTA	1:1000	Life Technologies
Claudin-7	Rabbit polyclonal	Ab27487	TRA, Dako	1:500	Abcam
Claudin-10	Rabbit polyclonal	PAD:ZMD.402, 38-8400	1mM EDTA	1:200	Life Technologies
ZO-1	Mouse monoclonal	ZO 1-1A12, 339100	1mM EDTA	1:200	Life Technologies
Occludin	Mouse monoclonal	OC-3F10, 33-1500	1mM EDTA	1:400	Life Technologies
E-cadherin	Mouse monoclonal	36/E-cadherin, 61081	TRS, Dako	1:500	BD Bioscience
Desmocollin-2	Rabbit polyclonal	Ab95967	TRS, Dako	1:500	Abcam
Desmoglein-2	Rabbit monoclonal	EPR6768	TRS, Dako	1:1000	Abcam
Connexin-43	Rabbit polyclonal	Ab11370	TRS, Dako	1:5000	Abcam