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Immunohistological characterization of intercellular junction proteins in rhesus macaque intestine

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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.

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.,

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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 et al., 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 μm. 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, Carpenteria, CA, USA) or 1 mM EDTA buffer pH 8.0 at 120 *circ*C for 4 min in a steam pressure decloaking chamber (Biocare Medical). The slides were then allowed to cool for 20 min, 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 min. 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 (PBS–FSG–Tx100) for 10 min 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 1 h 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 (Figs. 1 and 2). Claudin 7 expression appeared to be more in the junction and lateral membrane of luminal epithelial cells vs. crypt epithelium i.e. gradient