Characteristics of Claudin Expression in Follicle-Associated Epithelium of Peyer's Patches: Preferential Localization of Claudin-4 at the Apex of the Dome Region

Hiroshi Tamagawa, Ichiro Takahashi, Mikio Furuse, Yuka Yoshitake-Kitano, Shoichiro Tsukita, Toshinori Ito, Hikaru Matsuda, and Hiroshi Kiyono

Department of Surgery (HT, TI, HM), Osaka University Graduate School of Medicine, and Department of Mucosal Immunology (HT, IT, HK), Research Institute for Microbial Diseases, Osaka University, Osaka, and Department of Preventive Dentistry & Host Defense (IT), Hiroshima University Graduate School of Biomedical Sciences, Hiroshima, and Department of Cell Biology (MF, YY-K, ST), Faculty of Medicine, Kyoto University Graduate School, Kyoto, Division of Mucosal Immunology (HK), Department of Microbiology and Immunology, The Institute of Medical Science, The University of Tokyo, and CREST, JST (HK), Tokyo, Japan

SUMMARY: Gut-associated lymphoreticular tissues, such as Peyer's patches and cecal patches, are important inductive sites for mucosal immune responses. As such, gut-associated lymphoreticular tissues may have an epithelial barrier different from that of villous epithelium. In this study, we investigated the immunohistochemical distribution of the claudin family and occludin in the follicle-associated epithelium (FAE) of Peyer's patches and cecal patches of murine intestine. Unique profiles of claudin-2, -3, and -4 and occludin expression were noted in the tight junctions of the FAE: claudin-4 was preferentially expressed in the apex region; claudin-2 was only weakly expressed on the crypt side of the FAE compared with stronger expression on the crypt side of villous epithelial cells; and claudin-3 and occludin were found throughout the dome. These unique expression patterns were present also in cecal patch FAE. We also found that claudin-4 expression in the FAE of Peyer's patches and cecal patches correlated with the presence of TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling)-positive apoptotic cells, and Peyer's patch-deficient mice exhibited expression patterns of claudin and occludin in villous epithelia similar to those in wild-type mice. We conclude that claudin-4 expression was preferentially associated with the dome region of FAE, the mucosal inductive site of the murine intestine. In that location it might correlate with the cell life cycle, help maintain the apex configuration of the dome, or be a factor favoring the uptake of antigens by the FAE. (*Lab Invest* 2003, 83:1045–1053).

The intestinal mucosa is covered with one layer of epithelium. Enterocytes are generated from stem cells in the crypts, become mature forms, and migrate to the tips of the villi. The cells, which are continually renewed, have a life span of 2 to 4 days (Creamer, 1967). To maintain homeostasis and preserve the integrity of the epithelial barrier, the intercellular junctional complex, which consists of tight junctions, adherens junctions, and desmosomes, is thought to facilitate appropriate communication between outside and inside environments. Of these mechanisms, tight junctions located at the most apical side play a central role in sealing the intercellular space on epithelial sheets (Anderson and Van Itallie, 1995; Schneeberger and Lynch, 1992; Tsukita et al, 2001). Several pro-

teins, mainly occludin (Furuse et al, 1993) and claudin (Furuse et al, 1998; Morita et al, 1999a), comprise the tight junctions. Various cells, organs, and parts of tissue have unique distributions of the various claudins. According to a recent report, claudin-2, -3, -4, and -5 are highly expressed in the rat intestine (Rahner et al, 2001).

Peyer's patches (PP) are an important gut-associated lymphoreticular tissue (GALT) and are critical in the priming of antigen-specific T helper 1/T helper 2 cells and IgA-committed B cells, with dissemination of the primed lymphocytes to distant mucosal sites for the generation of antigen-specific IgA immune responses. Like other secondary lymphoid tissues, PP contain all of the necessary lymphoreticular cells, including APCs (macrophages and dendritic cells), lymphocytes, and supporting stromal cells for the induction of antigen-specific humoral and cellular immunity (McGhee and Kiyono, 1999). One of the unique features of this lymphoid tissue is the presence of an epithelium, called follicle-associated epithelium (FAE), which covers the dome of the patches (Owen,

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Address reprint requests to: Dr. H. Kiyono, Division of Mucosal Immunology, The Institute of Medical Science, The University of Tokyo, 4-6-1 Shirokanedai, Minato-ku, Tokyo, 108-8639 Japan. E-mail: kiyono@ims.u-tokyo.ac.jp

1977; Owen et al, 1991). Furthermore, M cells, one of the major components of the FAE, are key antigen-sampling cells for the delivery of orally encountered antigens to the underlying APCs (Neutra et al, 1996).

Some intestinal bacteria, eg, *Salmonella*, can invade the intestinal tract through M cells (Kohbata et al, 1986; Savidge et al, 1991). Two possible pathways for the uptake of these and other luminal antigens have been suggested: a transcellular pathway and a paracellular pathway (Atisook and Madara, 1991; Kops et al, 1996). Some kinds of *Salmonella* reportedly can invade also through villous epithelial cells (Niedergang et al, 2000; Vazquez-Torres et al, 1999); this invasion may be mediated by submucosal dendritic cells, which possess dendrites that extend toward the intestinal lumen between the epithelial cells (Rescigno et al, 2001). Claudin-1 (and also occludin) seem to be critical for the maintenance of the tight barrier between enterocytes and dendritic cells. In addition to involvement in the formation of tight junctions, the claudin family functions as a receptor for microorganism-derived molecules. For example, *Clostridium perfringens* enterotoxin binds to claudins, especially to the second loop of claudin-3 and -4 (Fujita et al, 2000; Sonoda et al, 1999). These phenomena suggest that bacteria-originated macromolecules can pass through paracellular pathways by modifying tight junctions. Further, it is also possible that the claudin family acts as a signaling receptor for communication between eukaryotic and prokaryotic cells.

This study was aimed at clarifying whether mucosal inductive tissues, such as PP and cecal patches, have tight junction strands in their FAE that differ from those in villous epithelia.

Results

Claudin and Occludin Expression in Murine Villous Epithelium

We first examined immunohistochemically the expression of claudin and occludin in murine intestinal epithelium. As expected from previous studies in rat intestine (Rahner et al, 2001), occludin was expressed throughout the mucosa of the small intestine (jejunum, Fig. 1A) and large intestine (cecum, Fig. 1B; rectum, data not shown). Among claudin family members, claudin-2 was expressed only in the gland crypts of small and large intestines, whereas claudin-3 was expressed in both the villi and the crypts. Claudin-4 was expressed sparsely in villous tips in the small and large intestines. Occludin, like claudin-3, was present on villi and crypts. Although claudin-2, -3, and occludin seemed to be localized preferentially on the apical side of the epithelial cells, claudin-4 was expressed stronger on the basolateral sides rather than on the apical sides. The different location of claudin staining pattern was clearly shown when the sections were costained with claudin family and ZO-1, a well-known tight junction marker (Fig. 1C). Although the expression of claudin-2, -3, and occludin were common in any parts of intestinal epithelia, our findings clearly

indicate the expression of claudin-4 by selective sites, not at every tip of villous epithelium.

Unique Characteristics of Claudin and Occludin Expression in FAE of PP and Cecal Patch

We next examined the expression of claudins and occludin in the FAE of PP (Fig. 2, A and B). Occludin and claudin-3 seemed to be expressed on the FAE and on the villous epithelium, from the crypt side of the FAE to the tip of the dome. Claudin-2 was faintly expressed in a restricted region near the crypt bases where claudin-2 was well expressed in case of villous epithelium. In contrast, claudin-4 was preferentially expressed in the FAE dome, and its expression was stronger than in villous tips. We also examined the distribution of claudins and occludin in the FAE of lymphoid patches present in the cecal wall (Fig. 2C). The expression patterns of these proteins were similar to those in PP: claudin-3 and occludin were expressed throughout the FAE, whereas claudin-2 was only faintly expressed at crypt bases, and claudin-4 was prominently expressed at the tops of the dome. In comparison to PP, cecal patches had large aggregates with overlying broad areas of epithelium, and claudin-4 was correspondingly extensively expressed.

Moreover, claudin-4 in the FAE was localized not only on the apical side but also on the basolateral side of epithelial cells (Fig. 2D), whereas in the villous tip it was exclusively expressed in the basolateral side. Costaining with ZO-1- and claudin-4-specific antibodies made the differences more distinguishable. ZO-1 was expressed only in the apical side of the epithelial cell through the dome of the PP. On the other hand, claudin-4 was found on the apical side as linear signal and vaguely on the basolateral side of the epithelial cells (Fig. 2D). Moreover the expression pattern of claudin-4 on the basal side of FAE was quite different from villous epithelia. These findings further illustrate that claudin-4 expression in FAE of PP was distinctively different from that of intestinal villous epithelium.

Claudin-4-Specific mRNA Expression by the FAE of PP

To confirm the immunohistochemical finding for the preferential expression of claudin-4 on the FAE dome (Fig. 2), we further examined the expression level of claudin-4-specific mRNA in epithelial cells isolated from PP and different locations of villous epithelium (Fig. 3). Thus, epithelial cells were isolated from the FAE dome and two distinct sites (tip and crypt regions) of jejunum. Total RNA was isolated from FAE (lane 1) and crypt (lane 2) and tip (lane 3) portions of villous epithelium for the semiquantitative RT-PCR analysis (Fig. 3). The level of occludin mRNA expression was similar among different specimens. However, the level of claudin-4 expression was strong in epithelial cells isolated from FAE of PP and the tip of villous epithelium when compared with the crypt of villous epithelium. These RT-PCR data also supported our immunohistochemical findings in which claudin-4

Figure 1.

Claudin and occludin expression in villous epithelia of murine jejunum and cecum. Immunohistochemical staining of claudin-2, -3, -4, and occludin in murine jejunum (A), cecum (B), and costaining with individual claudin (FITC) and ZO-1 (Cy-3) in jejunum (C). Specimens were cryosectioned (6 μ m) and stained with primary polyclonal antibodies specific for the respective claudin as well as occludin and ZO-1-specific mAbs, followed by secondary antibodies conjugated with FITC or Cy-3. Claudin-2 is expressed in the crypt region of jejunum and cecum, whereas claudin-3 is expressed in both crypts and villi. Claudin-4 is present sparsely on villous tip cells in the small and large intestines. Occludin, like claudin-3, is expressed by both crypt cells and villous cells. C, Claudin-2, claudin-3, and occludin are expressed on the apical side of the epithelium, but claudin-4 is expressed on the basolateral sides and on the apical sides of epithelial cells. Yellow arrowheads indicate examples of claudin-4-positive epithelial cells.

expression was associated with the FAE dome of PP and the tip of villous epithelium.

Possible Association Between Claudin-4 Expression and Apoptosis of Epithelial Cells and FAE of GALT

The localization of claudin-4 in villous tip epithelium and the dome FAE of PP and cecal patches reminded us of the distribution of apoptotic cells in these tissues. Thus, we performed TUNEL (terminal deoxynu-

cleotidyl transferase-mediated dUTP nick-end labeling) staining for the detection of apoptotic cells in the villous epithelium and GALT. Jejunal epithelium had a few TUNEL-positive cells at the apices of villi (Fig. 4A). TUNEL-positive cells were more frequently found in the FAE of PP and cecal patches. Thus, the distribution of TUNEL-positive cells was well correlated with that of claudin-4 in these sites (Fig. 4B).

To further confirm this immunohistochemical observation, a quantitative analysis for the evaluation of

Figure 2.

Claudin expression in Peyer's patches (PP) and cecal patches. Immunohistochemical staining of claudin-2, -3, -4, and occludin in murine PP in jejunum (A; low magnification, B; high magnification) and cecal patches (C). A and B, Claudin-2 is just faintly expressed in the restricted region near the crypt. Claudin-3 and occludin are expressed through the follicle-associated epithelium (FAE). Claudin-4 is preferentially expressed in the tip of the FAE dome compared with villous epithelium. C, The expression patterns of the claudins and occludin in the FAE are similar between PP and the cecal patches. Yellow arrowheads indicate examples of claudin-4-positive epithelial cells. D, Costaining of PP dome epithelium (FAE) with claudin-4- and ZO-1-specific antibodies. ZO-1 is expressed only in the apical side of the PP epithelia (left column). Claudin-4 is expressed in the apical side as linear signal and is found vaguely in the basolateral side of the epithelial cell (center column). Red (Cy-3) represents ZO-1, green (FITC) represents claudin-4, and the merged picture is also shown (right column).

Figure 3.

Semiquantitative RT-PCR for the expression of occludin- and claudin-4-specific mRNA by PP and villous epithelial cells. Epithelial cells were isolated from tip and crypt regions of jejunum and FAE of PP by means of a mechanical isolation manner followed by MACS cell sorting. Total RNA was isolated from FAE (lane 1), crypt side of villi (lane 2), and tip side of villi (lane 3). The cDNA amount used for each PCR was equalized by control gene (β -actin). The amplification fragments were visualized on 1.8% agarose gel using ethidium bromide. The levels of occludin-specific mRNA expression are similar among different three samples; however, the level of claudin-4 expression is stronger in FAE of PP and in the villous tip when compared with the villous crypt.

apoptotic cells and claudin-4-positive cells was performed (Fig. 4C). In the top part of the villous epithelium, the frequency of claudin-4-positive cells was $1.6 \pm 0.8\%$, whereas in the bottom part of the villi, claudin-4-positive cells were not found. On the other hand, the frequency of TUNEL-positive epithelial cells was $0.8 \pm 0.32\%$ in the upper villi and $0.1 \pm 0.06\%$ in the lower villi. In terms of the FAE of PP, however, the frequency of claudin-4-positive cells was $2.1 \pm 0.6\%$ in the bottom half and $21.6 \pm 5.2\%$ in the top half of the FAE. The frequency of TUNEL-positive cells was $0.9 \pm 0.3\%$ and $2.6 \pm 1.2\%$ in the bottom and top half of the FAE, respectively. Thus, it was shown that FAE of PP had high frequency of both claudin-4- and TUNEL-positive cells; in particular, claudin-4 was expressed preferentially in the top area of the FAE of PP.

PP-Null and Wild-Type Mice Had Similar Patterns of Claudins and Occludin Expression

Next we asked whether the intestines of PP-null mice and wild-type mice have similar or different expressions of claudin and occludin in the intestinal epithelium. We prepared three different kinds of PP-null mice: lymphotoxin α -deficient ($LT\alpha^{-/-}$), cytokine common γ chain-deficient ($C\gamma^{-/-}$), and NIK-deficient (aly/aly) mice. We found no obvious difference in the

Figure 4.

Relationship of claudin-4 expression and TUNEL (terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling) staining. TUNEL staining of jejunal villi and the FAE of PP. A, In the jejunum, a few TUNEL-positive cells (red arrows) are occasionally present in villous tips, where claudin-4 expression also is present. B, Claudin-4 expression in the FAE of PP is present in the top area of the domes, corresponding to the presence of TUNEL-positive cells. C, Frequency of claudin-4-positive cells and TUNEL-positive cells per villus or per FAE of PP. These FAE has high frequency of both claudin-4- and TUNEL-positive cells; in particular, claudin-4 is expressed preferentially in the top area on the FAE of PP. Data are shown as mean value $\pm$ SEM.

distribution of the tight-junction components in either the small intestine (Fig. 5) or large intestine (data not shown) between the various PP-null mice and wild-type mice. We looked especially carefully for a minor difference in claudin and occludin expression in the proximal jejunum and distal ileum of the PP-null mice because PP are most prevalent at these sites in normal mice, but we found no difference.

Discussion

In this study, we examined the distribution of claudin-2, -3, -4, and occludin in murine intestine and the GALT including PP and cecal patches. The results of our various experiments are summarized in Table 1. The major and potentially important finding was the preferential expression of claudin-4 in the dome area of the GALT FAE. In contrast, the expression pattern of other claudins and occludin was similar to that of villous epithelia. In contrast to its sparse expression in the apices of intestinal villi, claudin-4 was prominently expressed in the FAE. Previous studies have shown possible heterogeneity of claudin expression among different locations of tissue or cell type (Furuse et al, 1998; Kiuchi-Saishin et al, 2002; Morita et al, 1999b,

Figure 5.

Claudin expression in villous epithelium of PP-null mice. Immunohistochemical staining of claudin-2, -3, -4, and occludin in the small intestine of PP-null mice. Three different kinds of PP-null mice were examined: lymphotoxin α deficient ($LT\alpha^{-/-}$) (A), common γ chain deficient ($C\gamma^{-/-}$) (B), and NIK deficient (aly/aly) (C). All of the specimens were prepared from cryosections of jejunum. There is no significant difference in the expression pattern of claudins and occludin in the PP-null mice and wild-type mice: claudin-2 is present in the crypts, claudin-3 and occludin throughout the glands, and claudin-4 only occasionally in villous tips. In the large intestine also, the expression of the claudins and occludin was similar in the PP-null and wild type mice (data not shown). Yellow arrowheads indicate examples of claudin-4-positive epithelial cells.

1999c), including a unique distribution in the rat gastrointestinal tract (Rahner et al, 2001). Those studies, though, did not analyze the distribution of claudin and occludin in GALT. The previous study reported that claudin-2 was expressed in the crypts of small and large intestinal epithelia, whereas claudin-3 was found in both crypts and tip regions of intestinal epithelia. Further, claudin-4 was expressed on the tip of villous epithelia of small and large intestines (Rahner et al, 2001). In murine intestinal epithelium, we found claudin-2 expressed in crypts, whereas claudin-3 was expressed in villi and crypts. And claudin-4 was expressed in the villous tips just occasionally, not in every tip. Thus, our data in murine intestines generally agree with the results obtained by the analyses of rat intestinal epithelia, except for the frequency of expression of claudin-4.

The similarity in location of claudin-4 in the epithelium overlying the tips of intestinal villi and the FAE of GALT suggests that claudin-4 has a role in the formation and maintenance of the dome or apex shape of the villi and FAE. Villous epithelial cells are generated in the crypts and proceed upward to the villous tips, and FAE cells similarly are generated in crypts and migrate up to the top of domes of PP and cecal patches, ie, in the hemispherical area. Thus, it seems possible that claudin-4 contributes to maintenance of the hemispherical configuration.

Another possibility is that the expression of claudin-4 is related to the phenomenon of apoptosis because we found a close association between the locations of claudin-4 and apoptotic cells in intestinal villi and the FAE of PP and cecal patches. Also, we

noted a correlation between the numbers of TUNEL-positive cells and the expression of claudin-4; claudin-4 was abundant in the FAE of cecal patches, where TUNEL-positive cells were numerous, but was sparse in the villous tips, where apoptotic cells are rare (Hall et al, 1994). We suggest that claudin-4 is involved in the process of peeling off epithelial sheets of apoptotic enterocytes. None of the previous studies examined a potential relationship between claudin expression and cell life cycle, including apoptosis. Another interesting observation in the present study was that claudin-2 in the jejunum and colorectum was associated with the cells located in the epithelial crypts, in contrast to the location of claudin-4 on the apices of villi and the FAE. Taken together, these findings suggest that intestinal epithelial cells may be able to alter their junctional molecules in accordance with their different life cycle stages.

Evidence from this study suggests that claudin-4 expression actually may be associated with loosening of intercellular junctions. The apices of intestinal villi and the FAE are sites where foreign material may enter or be sampled by the gut. For example, some pathogenic microorganisms can invade through the epithelial cells in intestinal villi via intraepithelial dendritic cells (Rescigno et al, 2001; Vazquez-Torres et al, 1999). Since the FAE of GALT, with its associated M cells, is an important antigen-sampling site for the initiation of immune responses (Owen, 1977; Owen et al, 1991), looseness of its intercellular junctions may favor the capturing of antigens. An interesting possibility is that claudin-4 expression contributes to the formation of relatively loose intercellular junctions.

A subset of thymus-derived T cells, ie, CD8 $\alpha\beta$ T cells, residing in PP can migrate to intestinal epithelia and become part of the intraepithelial lymphocyte (IEL) pool (Cepek et al, 1994; Shaw et al, 1998). The IELs and their neighboring epithelial cells reciprocally regulate their development and growth via cytokines and corresponding receptor signaling (Fujihashi et al, 1996; Inagaki-Ohara et al, 1997; Yamamoto et al, 1998). Hence, we considered the possibility that some subsets of IEL derived from PP help the regulation of claudin and occludin expression in the intestinal epithelium. However, we found that claudin expression in the villous epithelium of PP-null mice was no different from that of wild-type mice, nor did preprogrammed sites for the development of PP have abnormalities in claudin or occludin expression. These findings suggest that PP-derived IELs are not involved in the regulation of claudin and occludin expression. Currently, we are attempting to determine whether thymus-independent gut-originated T cells have such a function.

Materials and Methods

Animals

C57BL/6 mice and lymphotoxin α -deficient mice ($LT\alpha^{-/-}$) were purchased from Japan CLEA (Tokyo, Japan). NIK-deficient mice (aly/aly) were purchased

Table 1. Summary for the Expression Pattern of Claudin-2, -3, -4, and Occludin in Murine Intestinal Epithelial and FAE of GALT

Animal	Tissue	Claudin-2	Claudin-3	Claudin-4	Occludin
C57BL/6	Jejunum	+ (crypt)	++	± (tip)	++
	Colorectum	+ (crypt)	++	± (tip)	++
	PP (FAE)	-/±	+	++ (tip)	+
	Cecal patch (FAE)	-/±	+	++ (tip)	+
LT $\alpha^{-/-}$	Jejunum	+ (crypt)	++	± (tip)	++
C $\gamma^{-/\gamma}$	Jejunum	+ (crypt)	++	± (tip)	++
aly/aly	Jejunum	+ (crypt)	++	± (tip)	++

-, no expression; ±, occasionally or weakly expressed; +, always expressed; ++, always and strongly expressed.

from SLC (Tokyo, Japan). Cytokine common γ chain-deficient mice (C $\gamma^{-/\gamma}$) were kindly provided by Dr. H. Ishikawa, Keio University, and were maintained in the Experimental Animal Facility at the Research Institute for Microbial Diseases, Osaka University (Osaka, Japan).

Preparation of Specimens

Mice were killed with 150 mg/kg body weight of ketamine. The whole gastrointestinal tract was then dissected and extensively washed with cold PBS, followed by immersion in Tissue-Tek OCT Compound (SAKURA Finetechnical Company, Ltd., Tokyo, Japan). The specimens were rapidly frozen in liquid nitrogen for cryosectioning. Cryosections were prepared on a Leica Cryostat CM3050S (Leica Microsystems, Wetzlar, Germany) at -20°C in $6\text{ }\mu\text{m}$ thickness. Sections were mounted on glass slides with APS coating (Matsunami Glass Ind., Ltd., Osaka, Japan) and postfixed in 95% ethanol at 4°C for 30 minutes followed by 1-minute acetone fixation. The slides were washed in PBS at 4°C for 10 minutes in preparation for staining.

Antibodies

Rabbit polyclonal anti-mouse claudin-2, -3, -4, and antibodies were raised as described (Furuse et al, 1998). These antibodies specific for the cytoplasmic domains of claudins and occludin were affinity purified on nitrocellulose membranes with glutathione-S-transferase fusion proteins of respective claudin proteins (Furuse et al, 1999; Morita et al, 1999a). Affinity-purified rabbit mAb against occludin (Zymed Laboratories, South San Francisco, California) was used for the detection of occludin expression. Rat mAb against ZO-1 (Chemicon, Temecula, California) was used for double immunohistochemical staining with other tight junctional antibodies.

Isolation of Villous Epithelial Cells and FAE of PP

Single-cell suspensions of epithelial cells were prepared by a mechanical and enzymatic dissociation method using type IV collagenase (Sigma, St. Louis, Missouri) as described previously (Fujihashi et al, 1996; Kawabata et al, 1997; Takahashi et al, 1997).

Briefly, after removal of PP, the intestine was opened longitudinally, washed thoroughly, and cut into small fragments. Tip side epithelial cells and IEL were removed from intestinal tissue by incubating in RPMI 1640 (Sigma) containing 2% fetal bovine serum, shaking vigorously, and filtering. After several repetitions of this procedure, the specimens were then minced and added to Joklik's modified medium containing collagenase. Crypt side epithelial cells and lamina propria lymphocytes were dissociated by stirring at 37°C . Then epithelial cells in respective specimens were isolated using the discontinuous density gradient procedure with Percoll (Amersham Pharmacia Biotech, Uppsala, Sweden). Furthermore CD3 ϵ -positive or CD19-positive or MHC class II-positive cells were eliminated by magnetic cell sorting (autoMACS; Miltenyi Biotec, Bergisch Gladbach, Germany). Similar procedures were also performed for the FAE of PP.

RT-PCR for Claudin-4- and Occludin-Specific mRNA

Total mRNA was isolated from MACS-sorted epithelial cells by using TRIzol reagent (Invitrogen, Carlsbad, California), treated with DNase I (Invitrogen), and reverse transcribed into cDNA using PCR buffer (Invitrogen), RNase inhibitor (Toyobo, Tokyo, Japan), oligo(dT)₁₆ (Invitrogen), Superscript II reverse transcriptase (Invitrogen), and dNTPs (Amersham Biosciences, Uppsala, Sweden) (Yamamoto et al, 1998). The mixture was incubated at 42°C for 120 minutes and then heated to 90°C for 5 minutes. After treatment with RNase H (Toyobo), the synthesized cDNA and a series of diluted standard oligonucleotides were quantified with a spectrofluorometer using an Oli-Green ssDNA Quantification Kit (Molecular Probes, Eugene, Oregon). PCR amplification from 10 ng of cDNA for each sample was performed with the Gene-Amp PCR System 9700 (Perkin-Elmer, Branchburg, New Jersey). RT-PCR was performed using primer pairs for occludin (forward, 5'-ATTATGGTGGCCGTGTCC-3'; reverse, 5'-GAGTAGGGCTTGTGCTTGT-3') and claudin-4 (forward, 5'-TGGATGAAGTGCCTGGTG-3'; reverse, 5'-GGTTGTAGAAGTCGCGGA TG-3') for 35 cycles (94°C , 45 seconds; 52°C , 1 minute; 72°C , 1 minute). These oligonucleotides were synthesized by Asahi Techno Glass (Chiba, Japan). β -actin PCR products were amplified by use of β -actin-specific primer

(forward, 5'-TAGATGGGCACA- GTGTGGG-3'; reverse, 5'-GGCGTGATGGTGGGCA- TGG-3'). The amplified products were separated by electrophoresis on 1.8% agarose gel and visualized with ethidium bromide.

Fluorescence Microscopy

Specimens stained with immunofluorescence reagents were examined and recorded under a fluorescence confocal laser scanning microscope (Micro Radiance MR/AG-2; Bio-Rad, Hercules, California), attached to an Olympus BX50 (Olympus Optical Company, Ltd., Tokyo, Japan).

Immunohistochemical Analyses

Immunohistochemical staining for different claudins and occludin was performed as previously described (Furuse et al, 1999; Morita et al, 1999a), with minor modification. Fixed cryosections were blocked in PBS containing 1% BSA (Sigma) for 30 minutes at room temperature. The sections were incubated with primary antibodies diluted approximately 1:1000 in the blocking buffer for 30 minutes at room temperature and then rinsed twice for 5 minutes each in PBS. The sections were further incubated for 30 minutes at room temperature with secondary antibodies: FITC-conjugated goat anti-rabbit IgG (Chemicon) or Cy-3-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, Pennsylvania). Sections were washed three times with PBS and mounted with Immunon Perma Fluor Aqueous Mountant (Thermo Shandon, Pittsburgh, Pennsylvania). As controls, sections were incubated with primary antibody or secondary antibody only. Specimens were observed using a fluorescence confocal microscopy.

TUNEL Staining

Apoptosis in the tissue section was detected by means of the TUNEL method, using ApopTag Fluorescein in Situ Apoptosis Detection Kit (Intergen, Purchase, NY) for fluorescent staining. Cryosections (6 μ m) were applied, and the following procedures were performed per protocol. Briefly, postfix was performed with a mixture of 67% volume of ethanol and 33% volume of acetic acid at -20°C for 15 minutes. After washing with cold PBS, the specimens were incubated with terminal deoxynucleotidyl transferase to label the DNA strand breaks for 1 hour at 37°C. Finally, the specimens were reacted with anti-digoxigenin antibody conjugated with FITC for 30 minutes at room temperature in the dark, followed by observation using fluorescence microscopy.

Scoring of Apoptosis and Claudin-4 Cells

Scoring the frequency of TUNEL-positive and claudin-4-positive cells was performed using confocal microscopy as previously described, with minor modification (Marshman et al, 2001). Longitudinal sections of crypts and villi were selected for scoring on the basis that, from the crypt to the tip, they were all in the

same section and the crypt lumen was visible. Starting at the base of the crypt column, the cells were examined and counted on both sides and the cells positive for apoptotic fragments (TUNEL) or claudin-4 positive cells were enumerated. For the elucidation of apoptotic cells and claudin-4-expressing cells in murine intestine, the enterocytes in the top one third (tip region) and bottom one third (crypt region) of 20 villi per animal were scored. For the PP, the frequency of apoptotic cells and claudin-4-positive cells was examined in the dome epithelium (or FAE). Selected sections of villi and PP were scored independently for a second time. Comparisons of the numbers of apoptotic and claudin-4-positive cells were analyzed using Student's *t* test. A *p* value < 0.05 was considered statistically significant.

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