Site differences of Toll-like receptor expression in the mucous epithelium of rat small intestine

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Summary. Toll-like receptors (TLRs) are known to recognize pathogen-associated molecular patterns and might function as receptors to detect microbes. In this study, the distribution of TLR-2, -4 and -9 were immunohistochemically investigated in the rat small intestine. As a result, TLR-2 was detected in the striated borders of villous columnar epithelial cells throughout the small intestine, except for the apices of a small number of intestinal villi. TLR-4 and -9 were detected in the striated borders of the villous columnar epithelial cells only in the duodenum. TLR-4-immunopositive minute granules were found in the apical cytoplasms of epithelial cells, subepithelial spaces and blood capillary lumina. TLR-2 and -4 were detected in the striated borders of undifferentiated epithelial cells and in the luminal substances of the intestinal crypts throughout the small intestine, but TLR-9 was not detected in the crypts throughout the small intestine. Only TLR-4 was detected in the secretory granules of Paneth cells in both the jejunal and ileal intestinal crypts. These findings suggest that duodenal TLRs might monitor indigenous bacteria proliferation in the upper alimentary tract, that TLR-2 might also monitor the proliferation of colonized indigenous bacteria throughout the small intestine, that the lack of TLR-2 at the villous apices might contribute to the settlement of indigenous bacteria, and that TLR-2 and -4 are secreted from intestinal crypts.

Key words: Epithelium, Immunohistochemistry, Rat, Small intestine, Toll-like receptors

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Introduction

In general, an enormous number of indigenous bacteria settle in animal alimentary tracts, and physiologically make a symbiotic relationship with the host (Salminen et al., 1995; Batt et al., 1996; Berg, 1996). The apices of the intestinal villi are the fundamental settlement sites of indigenous bacteria, and also the shedding sites of effete epithelial cells in late apoptotic stage in the small intestine (Chin et al., 2006; Inamoto et al., 2008ab; Yamamoto et al., 2009). Therefore, the stable settlement of indigenous bacteria is considered to be maintained by the delicate balance between the proliferation rate of the bacteria and the exfoliation rate of the apoptotic epithelial cells (Qi et al., 2009b). The proliferation of indigenous bacteria in the alimentary tract is regulated by various host defenses, such as physical and chemical elimination from epithelial cells themselves, a thick mucous layer, digestive enzymes and secretion of several bactericidal substances (Batt et al., 1996; Didierlaurent et al., 2006; Inamoto et al., 2008ab). In the rat ileum, the intestinal villi immediately shorten at the initial stage of indigenous bacteria proliferation in the intervillous spaces, and the migration of villous columnar epithelial cells accelerates in the intestinal villi in whose intervillous spaces indigenous bacterial colonies expand over the 50% position of the villous length from the villous tips. From these findings, it has been speculated that special membrane receptors for the detection of indigenous bacteria proliferation exist on the surface of the intestinal villi (Qi et al., 2009a). However, it remains unknown which receptor detects the proliferation of indigenous bacteria.

Recently, the Toll-like receptor (TLR) family has

been introduced as receptors recognizing pathogen-associated molecular patterns (PAMPs), constituents of pathogens, such as bacteria, virus, protozoa or fungi (Gleeson et al., 2006; Kawai and Akira, 2007). The TLR family consists of more than 13 members (Kawai and Akira, 2007). In general, TLR-2, -4 and -9 are the ones that recognize bacterial components. Specifically, TLR-2 recognizes peptidoglycan, lipoproteins and lipoteichoic acids, contained in the cell wall of bacteria, TLR-4 recognizes lipopolysaccharides (LPS), contained in the cell wall of gram-negative bacteria, and TLR-9 recognizes non-methylated CpG DNA, abundantly contained in bacterial DNA (Akira et al., 2006; Gleeson et al., 2006; Netea et al., 2006). TLRs are mainly expressed by various immunocompetent cells, such as macrophages and dendritic cells in animal tissues. The recognition of PAMPs by TLRs leads to the activation of innate immune systems (Akira et al., 2006; Netea et al., 2006). The expression of TLRs in intestinal epithelial cells has also been investigated *in vitro* (Cario et al., 2000, 2002; Abreu et al., 2001; Ruemmele et al., 2002). However, the detailed distribution of TLRs in the epithelium of the small intestine has not yet been clarified *in vivo*. In this study, we immunohisto-chemically investigated the distribution of TLR-2, -4 and -9 in the mucous epithelium of the rat small intestine.

Materials and methods

Animals

Seven male Wistar rats aged 7 weeks (Japan SLC Inc., Hamamatsu, Shizuoka, Japan) were maintained under conventional laboratory housing conditions. They were permitted free access to water and food (Lab MR Stock, Nosan Corp., Yokohama, Kanagawa, Japan). The animal facility was maintained under conditions of a 12-hour light/dark cycle at $23\pm1°C$ and 50-60% humidity. Clinical and pathological examinations detected no signs of disorder in any animals. This experiment was approved by the Institutional Animal Care and Use Committee (Permission number: 19-05-07) and carried out according to the Kobe University Animal Experimentation Regulations.

Tissue preparation

Seven rats were intravascularly perfused with 4% paraformaldehyde fixative (4% PFA; pH 7.4) after euthanasia with an overdose peritoneal injection of pentobarbital sodium (Kyoritsu Seiyaku, Tokyo, Japan). After perfusion, the small intestine was divided into three segments, the duodenum, jejunum and ileum, and small tissue blocks, about 5 mm in width, were removed from each intestinal segment. All tissue blocks were immersion-fixed in cold 4% PFA for 6 hours at 4°C, then were snap-frozen in liquid nitrogen with reference to the embedding method described by Barthel and Raymond

(1990). Sections, 4 μm in thickness, were cut using a Coldtome HM505E (Carl Zeiss, Jena, Thüringen, Germany) and were placed on slide glasses precoated with 0.2% 3-aminopropyltriethoxysilane (Shin-Etsu Chemical Co., Tokyo, Japan).

Immunohistochemistry

The detection of antigens was conducted using the indirect method of enzyme immunohistochemistry. Briefly, after rinsing with 0.05% Tween-added 0.1 M phosphate buffered saline (TPBS; pH 7.4), the sections were immersed in absolute methanol and 0.5% H_2O_2 for 30 min, respectively. Three TPBS rinses were performed after all preparation steps to remove any reagent residues. Following blocking with 0.06% normal wild yellowtail serum (prepared in our laboratory) for 1 hour at room temperature (r.t.), the sections were reacted with anti TLR-2, -4 or -9 goat IgG (diluted at 1:100; Santa Cruz Biotechnol., Santa Cruz, CA, USA) for 18 hours at 4°C. Then the sections were incubated with horseradish peroxidase-conjugated anti goat IgG mouse IgG (diluted at 1:200; Chemican International, Billerica, MA, USA) for 1 hour at r.t.. Finally, the sections were incubated with 3,3'-diaminobenzidine (Dojindo Lab., Mashiki, Kumamoto, Japan) containing 0.03% H_2O_2, and were counterstained with methyl green. Control sections were incubated with TPBS or non-immunized goat IgG instead of the primary antibody.

Evaluation of immunoreactivity

In each immunoreaction with different primary sera, the intensity of the immunoreaction was evaluated in comparison with negative control sections. Briefly, an intensity equal to that in the negative control sections was estimated at "negative" and the highest intensity was estimated at "strong". In addition, intermediate intensities were categorized as "weak".

Quantitative histology

Forty intestinal villi were randomly chosen from two types of ileal sites: where indigenous bacteria largely settle and where they hardly settle, respectively. Then, the relative frequency of intestinal villi with TLR-2-negative striated borders in villous apices was quantified from each group. Furthermore, 30 intestinal villi were randomly chosen from two groups of ileal villi: those having apices with and without TLR-2-negative striated borders, respectively. Then, the relative frequency of intestinal villi with indigenous bacterial adherence to their apices was quantified from each group. The adherence of indigenous bacteria to the epithelial cell membranes in the villous apices was confirmed, from the basis of the studies in our laboratory (Inamoto et al., 2008b; Qi et al., 2009a; Yamamoto et al., 2009), under a light microscope using oil-immersion lens with 100-fold magnification.
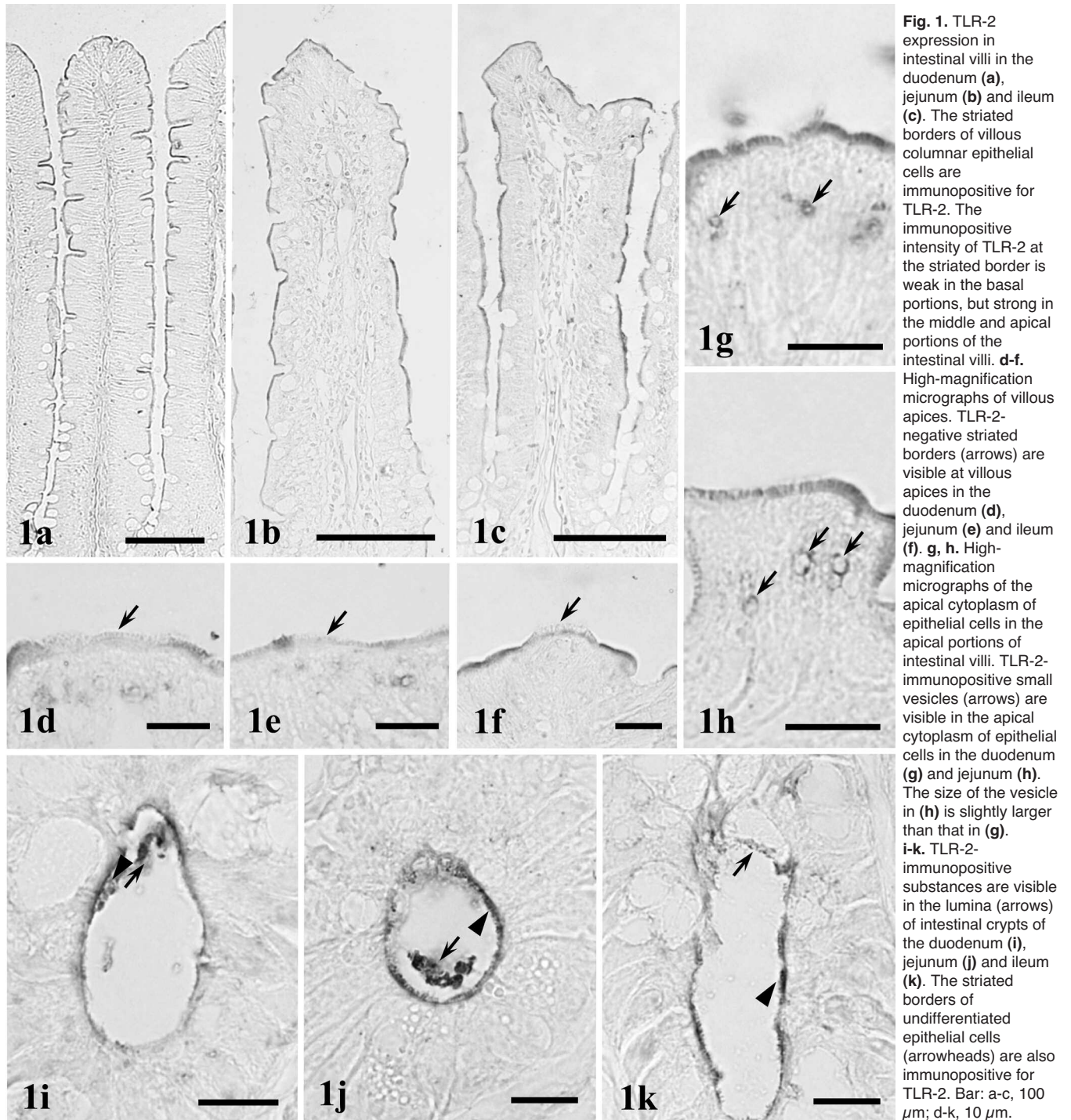
Data are presented as means ± standard deviations. Pearson's Chi-square test was employed in statistical analysis. P values less than 0.05 were considered statistically significant.

Results

Distribution of TLR-2

TLR-2 was detected in the striated borders of villous columnar epithelial cells of the intestinal villi throughout

Fig. 1. TLR-2 expression in intestinal villi in the duodenum **(a)**, jejunum **(b)** and ileum **(c)**. The striated borders of villous columnar epithelial cells are immunopositive for TLR-2. The immunopositive intensity of TLR-2 at the striated border is weak in the basal portions, but strong in the middle and apical portions of the intestinal villi. **d-f.** High-magnification micrographs of villous apices. TLR-2-negative striated borders (arrows) are visible at villous apices in the duodenum **(d)**, jejunum **(e)** and ileum **(f)**. **g, h.** High-magnification micrographs of the apical cytoplasm of epithelial cells in the apical portions of intestinal villi. TLR-2-immunopositive small vesicles (arrows) are visible in the apical cytoplasm of epithelial cells in the duodenum **(g)** and jejunum **(h)**. The size of the vesicle in **(h)** is slightly larger than that in **(g)**. **i-k.** TLR-2-immunopositive substances are visible in the lumina (arrows) of intestinal crypts of the duodenum **(i)**, jejunum **(j)** and ileum **(k)**. The striated borders of undifferentiated epithelial cells (arrowheads) are also immunopositive for TLR-2. Bar: a-c, 100 μm; d-k, 10 μm.

the small intestine. The immunopositive intensity of TLR-2 at the striated border was weak in the basal portions, but strong in the middle and apical potions of intestinal villi. TLR-2 was absent from the striated borders of some epithelial cells in a small number of villous apices (Fig. 1). Intestinal villi whose apices possessed TLR-2-negative striated borders were significantly more frequent in the group of ileal villi from the sites with the large settlements of indigenous bacteria than in the group of ones from the sites with scarce or no settlement of indigenous bacteria. On the other hand, adherence of indigenous bacteria was significantly more frequent in ileal villi with TLR-2-negative striated borders at the villous apices than in those without TLR-2-negative ones (Fig. 2). Small TLR-2-immunopositive vesicles were found in the apical cytoplasm of epithelial cells in the apical portions of intestinal villi throughout the small intestine, but the intestinal villi with TLR-2-immunopositive vesicles were more frequent in the duodenum and jejunum than in the ileum. The size of the vesicles in the jejunum was slightly larger than those in the duodenum and ileum (Fig. 1).

TLR-2 was also detected in the luminal substances of intestinal crypts and the striated borders of undifferentiated epithelial cells throughout the small intestine. These TLR-2-immunopositive intestinal crypts were more frequent in the jejunum and ileum than in the duodenum. TLR-2 was detected in the microvilli of some Paneth cells, but not in their secretory granules (Fig. 1).

Cell membranes and cytoplasm of migrating cells were negative for TLR-2 in the villous epithelium and rarely immunopositive in the lamina propria throughout the small intestine.

Distribution of TLR-4

In the duodenum, TLR-4 was detected in the striated borders of villous columnar epithelial cells, but negative striated borders were occasionally and randomly found in epithelial cells of the intestinal villi. Both their immunopositive intensity and the number of immunopositive epithelial cells gradually increased toward the villous apices. In the jejunum and ileum, TLR-4 was negative in the striated borders in almost all intestinal villi, but was occasionally detected in the striated borders of the villous apical portions (Fig. 3). In the jejunum from three animals, TLR-4 occasionally showed homogeneous immunopositive expression in the apical cytoplasm of epithelial cells in the apical portions, but was not detected in other portions of intestinal villi. In the three animals, such cytoplasmic immunoreactivity for TLR-4 was not detected in the ileum and duodenum. In the other animals, the homogeneous cytoplasmic immunopositivity for TLR-4 was never detected in villous columnar epithelial cells anywhere in the small intestine. In all animals, numerous TLR-4-immunopositive minute granules were found in the apical cytoplasm of epithelial cells, subepithelial spaces and blood capillary lumina (Fig. 3).

TLR-4-immunopositive substances were found in the lumina of intestinal crypts throughout the small intestine. The striated borders of undifferentiated epithelial cells were TLR-4-immunopositive in some intestinal crypts, more frequently in the duodenum and jejunum than in the ileum. Several Paneth cells possessed TLR-4-immunopositive secretory granules in both the jejunum and ileum, but not in the duodenum. These TLR-4-immunopositive Paneth cells were more frequent in the ileum than in the jejunum. TLR-4-
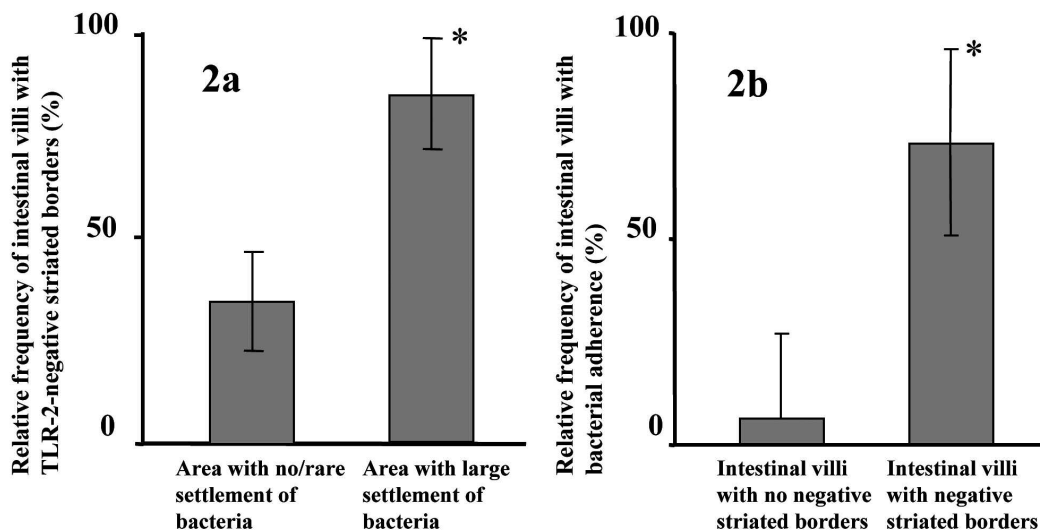

Fig. 2. a. Relationship between the settlement of indigenous bacteria and the relative frequency of intestinal villi with TLR-2-negative striated borders of villous apices in the ileum. **b.** Relationship between the relative frequency of intestinal villi with bacterial adherence to villous apices and the existence of TLR-2-negative striated borders in their apices. Asterisk, P<0.01. Each value represents means ± S.D.

immunopositive secretory granules differed in number among Paneth cells (Fig. 3).

TLR-4-immunopositive expression was found in the cell membranes or cytoplasm of migrating cells in the lamina propria of the intestinal villi throughout the small intestine. The immunopositive migrating cells were
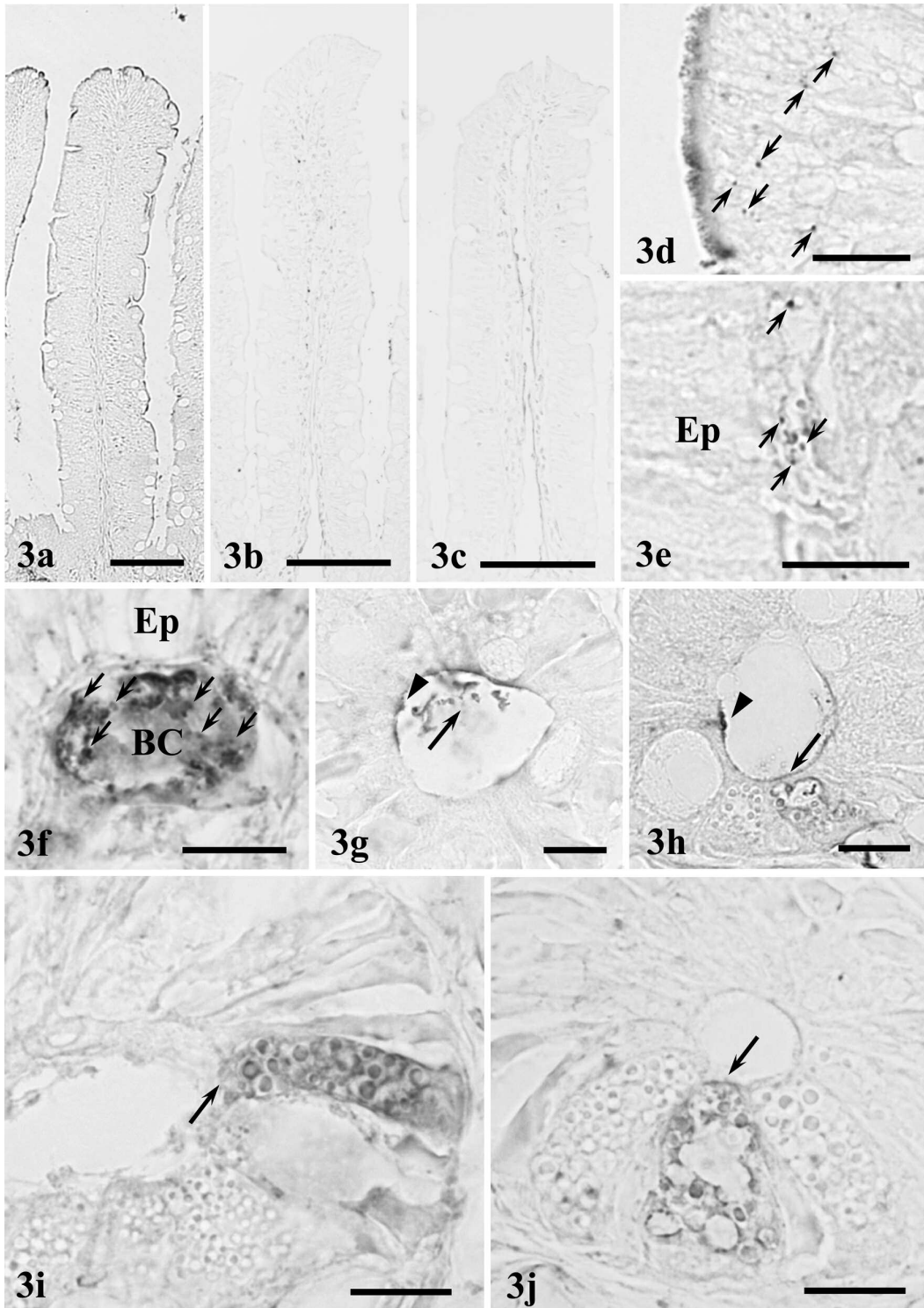
Fig. 3. a-c. The distribution of TLR-4 in the intestinal villi of the duodenum **(a)**, jejunum **(b)** and ileum **(c)**. **a.** In the duodenum, the striated borders of epithelial cells from the basal to apical potions of intestinal villi are immunopositive for TLR-4. **b, c.** In the jejunum and ileum, the striated borders throughout the intestinal villi are negative for TLR-4. **d-f.** High-magnification micrographs of the apical portions of the intestinal villi. Many TLR-4-immunopositive minute granules are visible in the apical cytoplasm of epithelial cells (**d**, arrows), the subepithelial spaces (**e**, arrows) and the lumen of subepithelial blood capillary (**f**, arrows) in the apical portions of intestinal villi. **g.** In the duodenal intestinal crypt, a luminal substance (arrow) and the striated borders of some undifferentiated epithelial cells are immunopositive for TLR-4 (arrowhead). **h.** In the jejunal intestinal crypt, the secretory granules of Paneth cells (arrow) and the striated borders of an undifferentiated epithelial cell (arrowhead) are immunopositive for TLR-4. **i, j.** In the ileal intestinal crypts, various numbers of TLR-4-immunopositive secretory granules are visible in the cytoplasm of Paneth cells. Many TLR-4-immunopositive secretory granules are visible in the cytoplasm of Paneth cells (**i**, arrow), but a few TLR-4-immunopositive secretory granules are visible in the cytoplasm of Paneth cells (**j**, arrow). BC, blood capillary; Ep, epithelial cells. Bars: a-c, 100 μm; d-k, 10 μm.

more localized in the jejunum than in the duodenum and ileum. Minute TLR-4-immunopositive granular substances were contained in the cytoplasm of migrating cells in the lamina propria of only the jejunum. No TLR-4-immunopositive migrating cells were found in the epithelium.

Distribution of TLR-9

In the duodenum, TLR-9 was detected in the striated borders of villous columnar epithelial cells of the intestinal villi, but was absent from those of some epithelial cells at a small number of villous apices. The TLR-9-immunopositive intensity of the striated borders gradually increased toward the apical portions of intestinal villi. In the jejunum and ileum, TLR-9 was negative in the striated borders of villous columnar epithelial cells in almost all intestinal villi, but occasionally detected in the striated borders at the apical portions of intestinal villi (Fig. 4). No TLR-9-immunopositive reaction was observed in the epithelial cytoplasm throughout the small intestine. No TLR-9 was detected in the intestinal crypts throughout the small intestine (Fig. 4).

TLR-9-immunopositive reactions were found in the cytoplasm of migrating cells in the lamina propria throughout the small intestine. The immunopositive migrating cells were more often observed in the jejunum than in the duodenum and ileum. Minute TLR-9-immunopositive granular substances were localized in the cytoplasm of migrating cells in the lamina propria of only the jejunum. In the epithelium, no TLR-9-
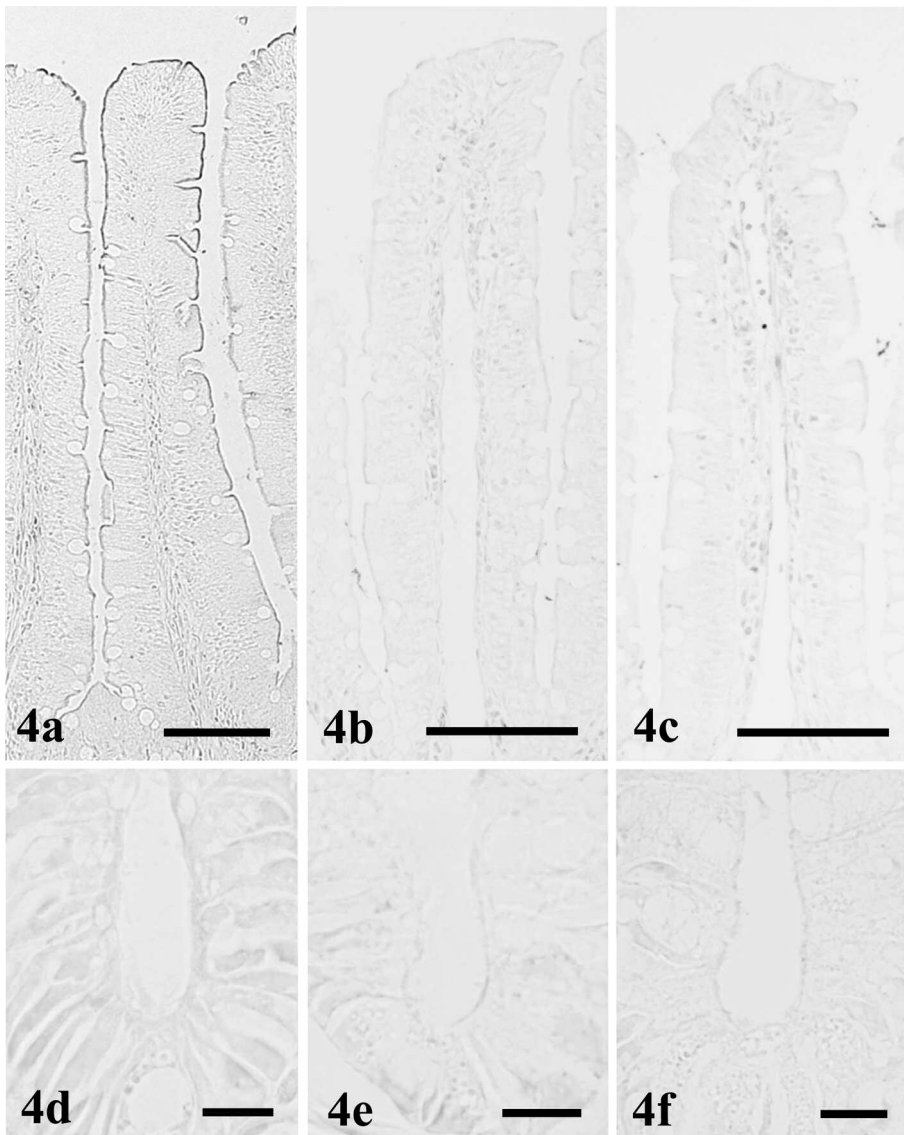
Fig. 4. The striated borders throughout the intestinal villi are immunopositive for TLR-9 in the duodenum **(a)**. The striated borders throughout the intestinal villi are negative for TLR-9 in the jejunum **(b)** and ileum **(c)**. TLR-9 positive immunoreaction is not seen in the intestinal crypts of the duodenum **(d)**, jejunum **(e)** and ileum **(f)**. a-c) Bars: a-c, 100 μm; d-f, 10 μm.

immunopositive migrating cells were found.

In all control sections, no positive immunoreactions were detected.

Discussion

A large number of indigenous bacteria colonize in the rat upper alimentary tract. The indigenous bacteria exclusively adhere to the dorsal surfaces of filiform papillae in the tongue and the corneal layer of the stratified squamous epithelium in the esophagus and nonglandular part of the stomach (Yamamoto et al., 2009). No mucosal lymphoid follicles exist in the rat upper alimentary tract (Koornstra et al., 1993; Yamamoto et al., 2009). The expression of TLR-2, -4 and -9 has not been reported on the surface of the rat stratified squamous epithelium, although the expression of TLR-2 and -4 has been reported in human keratinocytes (Baker et al., 2003; Pivarcsi et al., 2003). In the present study, all TLRs for bacterial components, TLR-2, -4 and -9, existed in the striated borders of the villous columnar epithelial cells in the duodenum. These findings suggest that duodenal TLRs might recognize their ligands from the upper alimentary tract to monitor the proliferation of indigenous bacteria in the upper alimentary tract.

In the rat small intestine, the fundamental settlement sites of indigenous bacteria are the apices of intestinal villi and the follicle-associated epithelium (Chin et al., 2006; Inamoto et al., 2008ab; Yamamoto et al., 2009). Indigenous bacteria proliferate into the intervillous spaces in the rat small intestine (Yamamoto et al., 2009). Intestinal villi become shorter in sites with the expansion of indigenous bacterial colonies in intervillous spaces over the 10% position of the villous length from the villous tips. Furthermore, the migration speeds of villous columnar epithelial cells significantly increase in sites with the expansion of indigenous bacterial colonies in intervillous spaces over the 20% position and reach the maximum over the 50% position of the villous length from the villous tips. From these findings, they speculate the existence of epithelial receptors recognizing the indigenous bacterial proliferation degree, such as TLRs, in the villous surfaces (Qi et al., 2009a). In the present study, TLR-2 was detected in the striated borders of villous columnar epithelial cells except for the villous apices throughout the small intestine. From this finding, TLR-2 of the intestinal villi might monitor proliferation of the colonized indigenous bacteria throughout the small intestine.

Several bacterial adhesion molecules, such as invasin, intimin and adhesin, were reported on the mucosal surfaces (Isberg and Van Nhieu, 1995; Frankel et al., 1996; Mouricout and Védrine, 2000). Adhesin are microbial lectins that selectively recognize glycoconjugates on the surface of intestinal epithelial cells (Mouricout and Védrine, 2000). Specific sugar expressions are restricted to the settlement sites of indigenous bacteria: that is, the striated borders of villous columnar epithelial cells in the late stage of apoptosis in the apices of rat intestinal villi, or the most luminal portions of other mucosal epithelium in the rat alimentary tract (Chin et al., 2007; Yamamoto et al., 2010). In the present study, TLR-2 was absent from the striated borders of some epithelial cells at the villous apices throughout the small intestine. The intestinal villi lacking TLR-2 in their apices were significantly more frequent in the sites with the large settlements of indigenous bacteria than in the sites with scarce or no settlement of indigenous bacteria in the villous apices. Furthermore, the intestinal villi with adherence of indigenous bacteria were significantly more frequent in the intestinal villi with TLR-2-negative striated borders at the villous apices than those with no TLR-2-negative ones. These findings suggest that the lack of TLR-2 contributes to the settlement of indigenous bacteria on epithelial cells in villous apices.

So far, the expression of TLRs in intestinal epithelial cells has been mainly investigated *in vitro* (Cario et al., 2000, 2002; Abreu et al., 2001; Ruemmele et al., 2002). The distribution of TLRs in intestinal crypts has not been histologically investigated so far. On the TLR-9, Ikeda et al. 2010 clarified that its mRNA level is very low in the intestinal epithelial cells by using RT-PCR technique. In the present study, TLR-9 was detected in the villous columnar epithelial cells of the duodenum, but not of the jejunum and ileum. The settlement of indigenous bacteria on the villous columnar epithelial cells increases distally in the rat small intestine (Yamamoto et al., 2009). Therefore, we suggest that the lack of TLR-9 might be one of the tolerance systems to the settlement of indigenous bacteria in the jejunum and ileum. On the other hand, TLR-4 mRNA has been detected in the crypt epithelial cells, but not in the epithelial cells of the intestinal villi in the human ileum by means of microdissection and RT-PCR methods (Wolfs et al., 2010). Furthermore, TLR-2 and -4 were reported to be expressed in HIEC lines, originating from the human fetal small intestinal crypt cells (Ruemmele et al., 2002). In the present study, TLR-2 and -4 were detected in undifferentiated epithelial cells, and the distribution of TLR-4 was mostly restricted in the intestinal crypts in both the jejunum and ileum. From these findings, using immunohistochemistry, we were able to demonstrate the findings from the previous reports with molecular biological methods or *in vitro*.

Recently, soluble forms of TLRs were detected in various secretions, such as human saliva, breast milk or blood plasma (LeBouder et al., 2003; Kuroishi et al., 2007; Zunt et al., 2009). In the present study, TLR-2 and -4 were detected in the luminal substances of the intestinal crypts throughout the small intestine. This finding suggests that TLR-2 and -4 are secreted from intestinal crypts, although cells secreting TLR-2 or -4, except for Paneth cells, could not be determined in the present study.

The intestinal epithelium is constantly exposed to various antigens derived from food or microorganisms,

and absorbs various ingested nutrients, such as amino acids, monosaccharides, fatty acids and so on. In contradiction to this established theory, however, many investigators have reported the phenomenon, *persorption*, by which intestinal epithelial cells transport macromolecules or particulates from the intestinal lumen to the hepatic portal blood (Yuji et al., 2006, 2007). LPS, which is the ligand of TLR-4, was detected in intraoperative samples of human portal blood and in the plasma of mice gavaged with dietary long-chain triglycerides (Jacob et al., 1977; Ghoshal et al., 2009). From these findings, it is speculated that intestinal epithelial cells absorb not only food-derived nutrients, but also bacterial components, such as LPS. Furthermore, the soluble form of TLR-4 was detected in human saliva (Zunt et al., 2009). In the present study, numerous TLR-4-immunopositive minute granules were found in the apical cytoplasm of epithelial cells, subepithelial spaces and blood capillary lumina. These findings might indicate that TLR-4-immunopositive minute granules are persorbed from the intestinal lumen into the subepithelial blood capillaries, and that TLR-4-immunopositive minute granules might be TLR-4-LPS complexes.

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