Porosity of the Epithelial Basement Membrane as an Indicator of Macrophage-Enterocyte Interaction in the Intestinal Mucosa

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Summary. The epithelial basement membrane of intestinal villi is perforated with numerous small pores, through which free cells in the lamina propria communicate with the enterocytes. This study was a comparative analysis of the pores in the basement membrane by SEM after removal of the gut epithelium with OsO₄ maceration. The porosity as represented by the area fraction of the pores varied along the baso-apical axis of villi in patterns specific for each animal species examined: consistent scantiness along the entire length of villi in mice, acute elevation in the second and third distal one-sixths of villi in rats, and gradual augmentation toward the villus tips in guinea pigs. Size distribution analyses of the pores indicated their heterogeneous enlargement in the regions of elevated porosity. Concomitant observation of lamina propria macrophages by histochemical labelings and by conventional TEM showed that the cells specifically clustered beneath the hyperporous basement membrane, with their thick processes penetrating it. The spatially-regulated patterns of perforation of the epithelial basement membrane indicate phase-specific interventions of lamina propria macrophages in the maturation or aging of enterocytes, which steadily proliferate in crypts and exfoliate at the villus tips.

Previous studies by transmission electron microscopy (TEM) have shown that the epithelial basement membrane in the intestinal villi is perforated with small pores through which free cells in the lamina propria are brought into direct contact with enterocytes (PALAY and KARLIN, 1959; DONNELLAN, 1965; TONER and FERGUSON, 1971). The distribution of these pores has been observed in the rat intestine by scanning electron microscopy (SEM) after extensive removal of the epithelium by OsO₄ maceration (HIGHISON and LOW, 1982; LOW and MCCLGAGE, 1984; MCCLGAGE and LOW, 1984). According to the SEM reports, porosity of the basement membrane is augmented in distal portions of villi except the apex, suggesting focalized interactions between free cells and enterocytes in the corresponding area (KOMURO, 1985; TAKAHASHI-IWANAGA and FUJITA, 1985). However, little information has been available for other species of animals concerning the perforation patterns of the basement membrane.

We have recently shown that macrophages in some species of animals aggregate in the lamina propria near the tip of intestinal villi, with their large processes protruding into the epithelium (IWANAGA et al., 1994). The cells were assumed to play certain roles in the disposal of aged enteroctyes, which complete their cell life at the villus tips (LEBLOND and MESSIER, 1958; QUASTLER et al., 1959). The macrophage behaviors varied among animal species: 1) an intraepithelial insertion of cytoplasmic projections and simultaneous incorporation of apoptotic enterocytes as observed in guinea pigs and monkeys (IWANAGA et al., 1992; HAN et al., 1993; IWANAGA et al., 1993); 2) a simple insertion of processes without phagocytic activities in rats (IWANAGA et al., 1994); and 3) the absolute retention of the entire cell body within the lamina propria in mice and some other mammalian species. These phenomena should be noted when analyzing species differences in the perforation of the basement membrane.

The present study focuses on the pores of the epithelial basement membrane in intestinal villi as an indicator of interactions between free cells, especially macrophages, and enterocytes. Sizes of the pores and their distribution along the entire baso-apical extent of intestinal villi were examined by SEM after removal of the epithelium with OsO₄...
maceration. Distribution and behavior of lamina propria macrophages were observed at different levels of villi by light microscopy after histochemical labeling of the cells. Conventional TEM observation was also performed in order to confirm topographical relationships between free cells and the pores. The present report concerns the mouse, rat, and guinea pig, representative species displaying the three different behavior patterns of lamina propria macrophages.

MATERIALS AND METHODS

Male dd mice weighing about 30 g, male Wistar rats weighing about 220 g and male Hartley guinea pigs weighing about 300 g were used in the present study. All the animals were raised under conventional conditions. Their livers and digestive tracts were devoid of any pathological signs on both macroscopic and microscopic inspection. Animals of each species were divided into three groups, each consisting of five individuals, and subjected to the three respective microscopic examinations described below. The animal experiments were conducted in accordance with the Guidelines for the Care and Use of Laboratory Animals, Hokkaido University School of Medicine. Observations are reported on the jejunum, the upper one third of the small intestine excepting the duodenum.

SEM observation

The animals were perfusion-fixed with a mixture of 1% glutaraldehyde and 1% paraformaldehyde in 0.1 M phosphate buffer, pH 7.3. Segments of the jejunum were immediately excised, cut into small pieces, and rinsed in 0.1 M phosphate buffer (pH 7.3) for 1 h. After rinsing, the tissue pieces were macerated in 0.1% OsO₄ in 0.1 M phosphate buffer (pH 7.3) at 20°C for about 48 h. The macerated specimens were conductive-stained by the tannin-osmium method according to Murakami (1974), dehydrated through a graded series of ethanol and critical-point dried using liquid CO₂. The dried specimens were coated with platinum-palladium in an ion-sputter coater, Hitachi E-1030, and examined in a Hitachi S-5000 N SEM at an acceleration voltage of 10 kV.

Light microscopic observation

Macrophages in the jejunum of the three animal species were stained for acid phosphatase. Detailed histochemical procedures have previously been described (Iwanaga et al., 1994). Briefly, frozen sections, 10 μm in thickness, were made from intestinal segments which had been perfusion-fixed with 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.3, and stained for the detection of acid phosphatase activity, according to Burnstone (1958).

Some additional tissue pieces of the rat jejunum fixed with paraformaldehyde were processed by immunohistochemistry for NO synthase (NOS), a macrophage marker, in order to observe fine processes of macrophages. A rabbit polyclonal antiserum against a synthetic peptide of rat macrophage NOS (77-105) (RY 114, Yanaihara Institute, Fujinomiya, Japan) was used for labeling. Paraformaldehyde-fixed cryostat sections were incubated with the antiserum diluted in 1:5000. The antigen-antibody reaction sites were visualized by the avidin-biotin-peroxidase complex (ABC) method, using a kit (Histofine, Nichirei, Tokyo, Japan).

Conventional TEM observation

The animals were perfusion-fixed with 2.5% glutaraldehyde buffered at pH 7.3 with 0.1 M cacodylate. The wall of the jejunum was cut into small squares measuring about 2 mm on each side, and immersed in the same fixative for 3 h. After rinsing in 0.1 M cacodylate buffer, pH 7.3, the specimens were post-fixed for 1.5 h in 1% OsO₄ buffered with 0.1 M cacodylate, dehydrated through a graded series of ethanol, and embedded in Epon 812 via propylene oxide. Ultrathin sections were made along the length of villi, stained with uranyl acetate and lead citrate, and examined under a Hitachi H-7000 transmission electron microscope at an acceleration voltage of 75 kV.

Fig. 1. Scanning electron micrographs showing intestinal villi which expose an epithelial basement membrane penetrated by numerous pores after the removal of enterocytes by OsO₄ maceration. a. Mouse. Pores in the basement membrane are homogeneously small. Arrow indicates an intestinal crypt containing a macerated residue of epithelial cells. b. Rat. Larger pores accumulate in upper half of villi, except at the very tip. Arrows orifices of crypts which have lost epithelial lining. c. Guinea pig. The perforation is prominent near tips of the villi. Arrows crypts devoid of epithelial lining. d. High magnification of the epithelial basement membrane in a rat. At the rims of pores, a thin superficial layer of amorphous matrix and underlying meshwork of collagen fibrils (arrows) are distinguishable. Cytoplasmic projections of a free cell protrude through one of the pores (asterisks). Bars: 50 μm (a–c), 1 μm (d).
Fig. 1. Legend on the opposite page.
Quantitative analysis

The entire height of intestinal villi—except their epithelial covering—was equally divided into six zones, which were numbered from 1 to 6 in a baso-apical order. For SEM measurements on pores, intestinal villi thoroughly exposing the epithelial basement membrane were chosen and recorded on micrographs at an original magnification of 2000×. Four villi per animal and a total of 20 for each animal species were examined. Area and number of pores per unit area of basement membrane (area fraction and numerical density) were calculated for each zone of a villus based on measurements in the sample area, 1600 μm² in total, on the micrographs. Data obtained from different villi in a given animal species were expressed as means ± SD. For analysis of pore size distributions, mean diameters were calculated for 200 pores randomly selected from each zone of villi in each animal species, and expressed as histograms.

For measurements on macrophages, jejunal tissue stained for acid phosphatase were observed by light microscopy with a ×20 objective. Five villi that had been sectioned along a longitudinal plane containing their most distal ends were selected from each of the five individuals in every group, giving a total of 25 villi for each animal species. The volume fraction of macrophages in each zone of lamina propria except for the central lacteals in villi was calculated, and expressed as a mean ± SD for each animal species.

The above-mentioned measurements of histological parameters were performed with a Zeiss image analyzer (IBAS, Carl Zeiss, Germany). For each animal species, regional variations in the area fraction and numerical density of pores in the basement membrane, and the volume fraction of macrophages in the lamina propria were assessed by the Kruskal-Wallis test and subsequently, by Scheffe’s F test.

Fig. 2. Area fractions of pores occupying the epithelial basement membrane of intestinal villi in mice (a), rats (b) and guinea pigs (c) are expressed as percentages, and numerical densities of pores in the basement membrane in mice (d), rats (e) and guinea pigs (f) as numbers per 100 μm². Zones 1 to 6 indicate the proximal to distal sixths of the entire height of villi except for the epithelial coverings, respectively. Bars: means ± SD. Inset in a. Data set on a different y-axis scale to improve visualization of regional differences in the porosity. Asterisks indicate significant differences detected by Scheffe’s F test (p < 0.05).
Fig. 3. Histograms showing size distributions of pores of the epithelial basement membrane in different zones of intestinal villi in mice (a–f), rats (g–l) and guinea pigs (m–r). The pore size has been classified by diameter. Zones 1 to 6 indicate the proximal to distal sixths of the entire height of villi except for the epithelial coverings, respectively.
RESULTS

SEM observation

After removal of the gut epithelium by osmication, intestinal villi revealed their cores of lamina propria, which were thin and leaf-like in shape, with a round end (Fig. 1a–c). The villus cores were invested by a basement membrane, which comprised a superficial amorphous layer of basal lamina, and a deeper collagen mesh of lamina fibroreticularis (Fig. 1d). Numerous small pores penetrated through the basement membrane. The area fraction of pores occupying the basement membrane ranged widely from 0.5 to 18% depending on the species as well as on regions of villi within a given species of animal (Fig. 2a–c). In contrast, the numerical density of pores was roughly constant; each 10 μm square of the basement membrane contained about 2 pores in the entire extent of villi in all the animal species examined (Fig. 2d–f). Epithelial basement membrane in crypts lacked any perforations.

In mice, the area fraction of pores was no larger than 1.2% along the entire height of villi (Fig. 2a). The pore sizes were homogeneously small, displaying a single-peaked distribution around 0.75 μm in every zone of the villi (Fig. 3a–f). In rats, the area fraction of pores elevated acutely up to 6–8% in zones 4 and 5 of the villi, while it dropped to 1.5–2% in the remaining zones (Fig. 2b). In the former zones, the pore size distributions pursued broad asymmetrical curves with some distended up to 2.5–4 μm, while in the latter, they showed symmetrical curves with a single-peak around 1 μm (Fig. 3g–i). In guinea pigs, the area fraction of pores gradually increased toward the villus tip (Fig. 2c). The pore size distributions exhibited a single acute peak around 1.5 μm in zones 1, 2, and 3, and gradually broadened toward the right with multiple peaks from zones 4 to 6 (Fig. 3 m–r). At zone 6, 20% of the pores exceeded 5 μm in diameter.

Light microscopic observation of macrophages

The acid phosphatase reaction specifically detected macrophages in jejunal mucosa in all the animals examined (Fig. 4). The cells displayed stellate shapes radiating some processes with an unstained nucleus at the center. They were always situated in the lamina propria, with some exceptions found in the epithelium covering the villus tips of guinea pigs. Lamina propria macrophages were more numerous in villi than in crypts.

Lamina propria macrophages in mice were randomly dispersed along the entire height of villi, displaying attenuated cell bodies and projections (Figs. 4a, 5a). In rats, macrophages were significantly accumulated at zones 4 and 5 (Figs. 4b, 5b). In these regions of the villi, the cells frequently issued a straight process deep into the epithelium. The intraepithelial processes were better visualized in immunohistochemical preparations labeled for NO synthase (Fig. 4d). In guinea pigs, macrophages aggregated toward the villus tips with a significant elevation in volume density at zones 5 and 6 (Figs. 4c, 5c). In these zones, portions of the cells or their entire cell bodies frequently invaded the epithelium (Fig. 4e). Those macrophages aggregated at villus tips exhibited large, rounded cell bodies amply filled with coarse granular materials.

TEM observation

Lamina propria of intestinal villi contained numerous macrophages, plasma cells, lymphocytes and eosinophils in all the animals examined, in accord with previous reports (Deane, 1964; Sawicki et al., 1977). The lamina propria communicated with the gut epithelium via small pores in the basement membrane. The pores occurred along the entire height of villi. Most pores were no larger than 1 μm in width, and obstructed by fine projections of the free cells and those of epithelial cells.

In mice, lamina propria macrophages displayed angular cell bodies with rather scanty cytoplasm (Fig. 6a). The cells occurred sparsely along the entire height of villi. None of them invaded the epithelium.

Lamina propria macrophages in rats enclosed larger amounts of cytoplasm than did those in mice (Fig. 6b). The former cells aggregated at a short distance below the villus tip, corroborating the light microscopic findings in zones 4 and 5 of the rat villi. The macrophages issued a thick process straight into the epithelium through pores in the basal lamina. Most pores in this region of villi were obstructed by the penetrating processes, and distended up to 2–4 μm. The macrophage processes lacked any phagocytic signs as reported previously (Iwanaga et al., 1994).

Lamina propria macrophages in guinea pigs revealed round cell bodies amply filled with large phagosomes (Fig. 6c). The cells accumulated toward the villus tip. Some macrophages at the tips of villi protruded labial processes into the epithelium, while others as a whole, entered the epithelium. Both cases incorporated apoptotic bodies of effete enterocytes into the phagosomes in accord with previous reports (Han et al., 1993). Most pores measured 2–5 μm in width in this region of the basement membrane.
Fig. 4 a–c. Intestinal villi stained for acid phosphatase in a mouse (a), rat (b) and guinea pig (c). Macrophages in the lamina propria and apical portions of epithelial cells display intensely positive reactions. Arrows in c indicate central lacteals. d. High magnification of an upper portion of a rat intestinal villus stained by immunohistochemistry for NO synthase. Macrophages labeled with the antibody occur immediately beneath the basement membrane with a thick process projected straight into the epithelium (asterisks). e. High magnification of an apical portion of a guinea pig villus stained for acid phosphatase. A portion of a lamina propria macrophage protrudes into the epithelium (arrow). Asterisks macrophages. Bars: 100 μm (a–c), 10 μm (d, e)
DISCUSSION

The present study is the first to quantitatively compare pores of the epithelial basement membrane in intestinal villi among different animal species. The porosity as represented by the area fraction of the pores varied along the baso-apical axis of the villi in patterns specific for each animal species examined: consistent scantiness along the entire height of the villi in mice, acute elevation in the second and third distal sixths comprising zones 4 and 5 of the villi in rats, and gradual augmentation toward the villus tip in guinea pigs. Present observations by histochemistry and by conventional TEM indicate the responsibility of lamina propria macrophages for regional elevations in the porosity; the cells aggregated specifically beneath the hyperporous regions of the basement membrane with their cytoplasmic projections protruded through large pores. The macrophage processes are believed to heterogeneously enlarge the pores which they penetrate, because the pore size distributions in the corresponding regions of villi display multiple-peaked curves broadening along the x-axis. Intraepithelial invasions of lamina propria macrophages in intestinal villi have been reported in rats by immunohistochemical labeling of the cells (Mayrhofer et al., 1983) and by conventional TEM (Komuro, 1985), although little attention has been directed to the spatial pattern of the occurrence of this phenomenon.

It has generally been accepted that the enterocytes display a gradient of maturation or aging along the height of villi as a result of their continual proliferation in crypts and extrusion at the villus tips (LeBlond and Messier, 1958; Quastler et al., 1959). The focalized aggregation and invasion of macrophages at a regular height of the villi in rats and guinea pigs indicate synchronous intervention of the cells in a certain phase of the enterocyte lifetime. We have previously shown that macrophage processes affect effete enterocytes near villus tips, deeply interdigitating with the cells in rats (Iwanaga et al., 1994), or engulfing apoptotic cell fragments in guinea pigs (Han et al., 1993). The present findings have confirmed that the former events in rats occur a short period before the cell extrusion, and the latter exactly at the time of cell death. On the other hand, recent experiments with cell culture have shown that macrophages modulate permeability of the gut epithelium with their cytokines, such as TNF-α, INF-γ, and IL-6 (for review see: Lewis et al., 1995). One would presume that the potential regulation of the epithelial function by macrophages may also occur phasespecifically along the time course of the enterocyte maturation, at least in the rat and the guinea pig.

Macrophages isolated from intestinal mucosa have been known to display heterogeneity with regard to their adherent properties, phagocytic activities, effects on mixed lymphocyte reaction, and expressions of macrophage antigens (Pavli et al., 1990; Bland and Kambarage, 1991). Nagashima et al.

![Figure 5](attachment:image.png)

**Fig. 5.** Volume fractions of macrophages occupying the lamina propria—except for the central lacteal—in mice (a), rats (b) and guinea pigs (c) are expressed as percentages. Zones 1 to 6 indicate the proximal to distal sixths of the entire height of villi except the epithelial coverings, respectively. Bars: means ± SD. Asterisks indicate significant differences detected by Scheffe’s F test (p<0.05).
REFERENCES


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(1996), using immunohistochemistry for various macrophage markers, have shown that human colonic mucosa contains two types of macrophages: certain phagocytic macrophages gathering immediately beneath the epithelium, and antigen-presenting dendritic cells (Steinman, 1991) dispersed in deeper regions. The former cells, and not the latter, incorporated apoptotic enterocytes, resembling those guinea pig macrophages observed by us. The species differences in macrophage behavior in intestinal villi might imply different cell subtypes dominating the tissue.

Previous TEM studies in the mouse and in some mammalian species demonstrated that lymphocytes frequently obstruct pores of the epithelial basement membrane in intestinal villi, suggesting the busy traffic of the cells across the tissue septum (Maeder and Landers, 1967; Marsh, 1975; Röpke and Everett, 1976; Komuro, 1985). In the present SEM observation of mouse villi, however, the basement membrane mostly lacked large pores which would allow such cell passage. In contrast, the basement membrane covering cryptopatches, which have recently been characterized as foci of generation and recruitment of the intraepithelial T lymphocytes in the intestinal mucosa (Kanamori et al., 1996), has been known to display numerous large pores containing lymphocytes even in the mouse (Saito et al., 1998). These findings suggest that, at least in this animal species, lymphocyte traffic through the basement membrane in villi, if any, is only occasional, followed by repair of the trace.

The present study has shown species-specific patterns of perforation of the epithelial basement membrane in intestinal villi, and their significance as an indicator of interaction between macrophages and enterocytes under normal conditions. Both morphological and immunological aspects of the cell interactions require elucidation for an understanding of the physiology and pathology of the intestinal mucosa in different experimental animals as well as in humans.


