Electron Microscope Observations on the Formation of Primitive Villi in Rat Small Intestine with Special Reference to Intercellular Junctions*

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Summary. Formation and fusion of intraepithelial cavities have long been considered an essential process in the histogenesis of the intestinal mucosa. By electron microscope observation of thin sections and freeze-fracture replicas of the small intestine of rat fetuses, we first demonstrated the initial steps in the formation of intraepithelial cavities: A focal tight junction (macula occludens) was formed in the abluminal part of the epithelium, after which the membrane of an intracellular cavity was fused with that of the focal tight junction to form an intercellular (intraepithelial) cavity enclosed by a zonula occludens.

The present study also revealed that gap junctions appeared and enlarged simultaneously with the formation of primitive villi and differentiation of absorptive cells. These gap junctions gradually came to be confined in the epithelium of intervillous regions where proliferation and differentiation of epithelial cells took place. Absorptive cells in villi rarely had gap junctions.

These results suggest that tight and gap junctions play important roles in the histogenesis of the intestinal mucosa, and in the proliferation and differentiation of epithelial cells.

The small intestinal mucosa undergoes dramatic remodeling during fetal development (HILTON, 1902; JOHNSON, 1910; CHO, 1931; SHAWDUNN, 1967; KLEIN and McKENZIE, 1983; NAKAMURA and KOMURO, 1983; TRAHAIR and ROBINSON, 1986). The mucosa is originally smooth and lined with a simple columnar epithelium. Prior to formation of primitive villi, undifferentiated epithelial cells rapidly proliferate to form a thick stratified epithelium, and the intestinal lumen is extremely narrowed. After this, primitive villi lined with a single layer of columnar cells are formed. Extension of the intestinal lumen into the stratified epithelium is an essential process for formation of villi along with mesenchymal invasion. Electron microscope observations on thin sections have suggested that this process is accomplished by the fusion of intraepithelial cavities formed in the thickened epithelium (MATHAN et al., 1972, 1976; MOXEY and TRIER, 1978; TOYOTA et al., 1989).

By adding new observations obtained from the freeze-fracture technique, MADARA et al. (1981) hypothesized the entire process as follows: 1) plaque-like secondary junctional complexes were formed in abluminal parts of the epithelium; 2) a secondary lumen (an intraepithelial cavity in the present study) appeared in the center of the secondary junctional complex; and 3) extension of the main intestinal lumen was accomplished by fusion of secondary lumina with the main intestinal lumen. However, their observations remain incomplete, especially in showing the initial formation of intraepithelial cavities. In the present study using thin section and freeze-fracture methods, we first demonstrated that a secondary tight junction (macula occludens) was formed between two cells in the abluminal part of the epithelium, and then an intraepithelial cavity opened within the secondary tight junction. In addition, we found that gap junctions appeared and enlarged during the formation of villi when intestinal epithelial cells began to differentiate.

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MATERIALS AND METHODS

The animals used in this study were fetuses of Wistar-KY rats on days 13-20 of gestation (vaginal plug = day 0). Developmental ages of the fetuses were determined according to CHRISTIE (1964). The duodenum, jejunum and ileum were removed from the fetuses, and cut into small tissue blocks in 2% glutaraldehyde fixative (0.1 M cacodylate buffer, pH 7.3) at room temperature. The blocks were then immersed in the same fixative for at least 2 h at 4°C.

For the thin section method, the tissue blocks were postfixed in 1% osmium tetroxide (0.1 M cacodylate buffer, pH 7.3) for 1 h, dehydrated by graded concentrations of ethanol and embedded in Epon 812 resin. Thick sections, cut on an ultramicrotome, were placed on glass slides and stained with 1% toluidine blue for light microscope examination. Thin sections were doubly stained with uranyl acetate and lead citrate and examined in a Hitachi H-500 electron microscope (Hitachi Ltd., Japan).

For the freeze-fracture technique, the tissue blocks were immersed in 40% glycerin solution after fixation with glutaraldehyde. The specimens, placed on a small piece of Nylon mesh, were mounted on a copper stage, cooled in liquid nitrogen and fractured at -170°C to -180°C in a JFD-7000 freeze-fracture device (JEOL Ltd., Japan). Platinum-carbon replicas were made without etching. After thawing, the tissues, together with the small piece of Nylon mesh, were placed on a larger piece of Nylon mesh and floated on filtered commercial bleach for 2 h in a Teflon dish. Replicas were washed in 20% Extram MAO2 neutral solution (Merck, Germany) for 1 to 2 h and distilled water for 30 min, mounted on copper grids and examined in the electron microscope at 100 kV.

RESULTS

General features of the developing intestinal mucosa

General structures of the intestinal epithelium of rat fetuses as observed by light and electron microscopy have been reported elsewhere (TOYOTA et al., 1989). Briefly, a simple columnar epithelium consisting of undifferentiated cells lined the smooth intestinal mucosa on days 13-14 of gestation. Neighboring epithelial cells were interconnected by the primary junctional complex in the vicinity of the intestinal lumen.

On days 15-16 of gestation, the epithelium appeared to be stratified and the intestinal lumen was extremely narrowed. In addition to the adluminal primary junctional complex, secondary junctional complexes were formed in the abluminal part of the epithelium. Many intraepithelial cavities enclosed by the secondary junctional complex also appeared. The intraepithelial cavity, first surrounded by two cells, was enlarged by mitotic division of surrounding cells.

On days 17-18 of gestation, the main intestinal lumen was reopened by fusion of intraepithelial cavities, mesenchyme began to invade into the thick epithelium, and primitive villi were formed. At the upper part of the primitive villi, the epithelium consisted of a single layer of differentiating columnar cells which projected many microvilli. On the other hand, the stratified epithelium still lined the lower part of primitive villi and intervillous regions, and

Fig. 1. Undifferentiated epithelial cells interconnected by primary tight junctions (T) in the duodenum on day 14. Tight junctional strands extend to apical plasmalemmas in some parts (arrows) and secondary tight junctions have formed between apical plasmalemmas of neighboring cells (arrowhead and inset). ×6,000, Inset: ×50,000
many intraepithelial cavities were seen in these regions.

On days 19–20 of gestation, primitive villi were further divided to form definitive villi, which rapidly increased in number and height. In differentiating absorptive cells lining the villi, microvilli became longer and more numerous to form a brush border. A few goblet cells were also seen. The stratified epithelium gradually decreased in the intervillous regions on these days. In the duodenum and jejunum, secondary junctional complexes and intraepithelial cavities rapidly decreased. In the ileum, these structures decreased one day later than those in the proximal part of the small intestine.

Mitotic figures were seen to be related to the main intestinal lumen and intraepithelial cavities over the whole epithelium on days 13–18 of gestation, but were confined to the lower part of villi and intervillous regions on days 19–20 of gestation.

Junctional complexes and intraepithelial cavities

As a typical junctional complex in the adult intestine (Farquhar and Palade, 1963), a primary junctional complex surrounding the lumen of the developing intestine consisted of a tight junction (zonula occludens), intermediate junction (zonula adherens) and desmosomes. The intermediate junction, however, was disposed within the tight junction rather than being an independent structure. By the freeze-fracture method, development of primary tight junctions varied greatly on days 13–14 of gestation (Fig. 1). Sometimes a tight junction consisted of only one to two junctional strands, which were often seen as linear arrays of incompletely fused particles. Some open-ended strands extended toward the cell base or to the apical plasmalemma, and focal tight junctions (maculae occludentes) formed between apical plasmalemmas.

Fig. 2. Primary and secondary (Type I) tight junctions accompany small gap junctions (arrows) in the ileal epithelium on day 15. The arrowhead indicates endocytosis. ×20,000

Fig. 3. A thick jejunal epithelium on day 18 possesses intraepithelial cavities and various types of secondary tight junctions (arrows). ×4,000
of neighboring cells. On later days, the number of tight junctional strands were increased to form a meshwork of rounded polygones (Fig. 2). Basally oriented open-ended strands persisted, but apically oriented strands and focal tight junctions were no longer seen on the apical plasmalemma.

In addition to the primary junctional complex circumscribing the main intestinal lumen, many secondary junctional complexes and intraepithelial cavities were found in the abluminal part of the thickened epithelium on days 14-18 of gestation (Fig. 3). Tight junctions in secondary junctional complexes (secondary tight junctions) were classified into the following five types: Type I: Small fragments of tight junctional strands interconnected neighboring cells (Fig. 2). Type II: A large focal tight junction (macula occludens) formed between two cells (Fig. 4). Type III: A large tight junction formed where three cells adjoined (Fig. 5). Here a ladder-like extension of tight junctional strands was seen along the adjoining line.

Fig. 4. Secondary tight junctions (Type II) in the jejunal epithelium on days 16 (B) and 17 (A). Opposing plasmalemmas fuse with each other at several points (arrowheads). The cytoplasm contains intracellular cavities (arrows) and small vesicles. ×27,000. B. A macula occludens and associated desmosomes (arrows) are clearly demonstrated in the freeze-fracture replica. ×30,000

Fig. 5. Secondary tight junctions (Type III) and accumulation of small vesicles in the adjacent cytoplasm. A. Microfilaments and bundles of intermediate filaments are abundant in the cytoplasm around the vesicles and along the tight junction in the jejunal epithelium on day 18. ×20,000. B. A ladder-like extension (arrow) of the tight junction is seen where three cells join in the duodenal epithelium on day 17. ×14,000
of three cells; this Type III was only occasionally found. Type IV: A tight junction (zonula occcludens) enclosing a small intraepithelial cavity formed between two cells (Fig. 6). Type V: A tight junction (zonula occcludens) enclosed an intraepithelial cavity surrounded by more than two cells (Fig. 7). A ladder-like extension of the tight junction was formed where the three cells joined. In Types IV and V, tight junctional strands tended to extend in various directions.

These five types of secondary tight junctions were formed asynchronously after day 14 of gestation, although there was a certain tendency in the incidence of each type during fetal development. On days 14-16 of gestation, Types I to III were sometimes seen. Type IV occurred less frequently and Type V could not be found. On days 17-18 of gestation, all types were frequently seen, Type V surrounding large intraepithelial cavities being the most conspicuous (Fig. 3). After day 19 of gestation, the incidence of secondary tight junctions decreased rapidly.

In the secondary junctional complex, microfilaments adhered along the cytoplasmic side of the tight junction, and desmosomes tended to be incorporated in the meshwork of the tight junctional strands (Figs. 4-6). Caveolae suggesting the endocytosis of lateral plasmalemmas were sometimes found in the vicinity of the secondary tight junction (Fig. 2). Moreover, epithelial cells occasionally contained conspicuous vacuoles which were limited by double membranes interconnected by tight junctional strands (Fig. 8).

In addition to the intraepithelial (intercellular) cavities described above, intracellular cavities filled with many microvilli were seen near the secondary junctional complex (Figs. 4, 9). Small vesicles, 0.1-0.2 μm in diameter, were aggregated in the cytoplasm near secondary junctional complexes and intracellular and intraepithelial cavities (Figs. 4-6, 9). These vesicles opened to intermicrovillous regions of intracellular and intraepithelial cavities. An intracellular cavity further opened to an intraepithelial cavity, and the latter to another intraepithelial cavity or to the main intestinal lumen (Fig. 9).

**Gap junctions**

On days 13-16 of gestation, small gap junctions were occasionally found. They were associated with a primary tight junction, with open-ended strands extending basally from it and with secondary tight junctions (Figs. 2, 6B). Gap junctions isolated from tight junctional strands were seen less frequently (Fig. 10A, B).

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**Fig. 6.** Secondary tight junctions (Type IV) and intraepithelial cavities (C) formed between two cells in the jejunum on days 15 (A, B) and 16 (C). A. Microvilli project into the cavity, and small vesicles are seen nearby. ×16,000. B. The tight junction accompanies small gap junctions (arrows) and desmosomes (arrowheads). ×28,000. C. A more developed tight junction extending junctional strands radially. A small macula occludens (arrow) is also seen. ×15,000
On days 17-20 of gestation, when primitive villi were formed and epithelial cells began to differentiate, gap junctions increased in association with the primary tight junction and secondary tight junctions of Types I to V. In addition, gap junctions isolated from tight junctional strands increased rapidly in number and size (Fig. 10C-E). After primitive villi were formed, gap junctions were found more frequently in the lower portion of the villi than in the upper portion. On day 20 of gestation, gap junctions tended to be located in intervillous regions.

These growing gap junctions often accompanied large particles 11-13 nm in diameter, with usual gap junctional particles being 8-9 nm in diameter. The large particles were distributed randomly (Fig. 10D) or arranged in hexagonal arrays (Fig. 10B, C). Even when these large particles was not so conspicuous, a small number of them would be located at the periphery of gap junctions (Fig. 10E).

**DISCUSSION**

The present study demonstrated that the development of tight and gap junctions is closely related to the formation of intestinal villi and differentiation of absorptive cells.

**Tight junctions and the formation of primitive villi**

The appearance of intraepithelial (intercellular) cavities, a common phenomenon, occurs when an epithelial cell mass having developed into connective tissue forms new lumina, such as the follicular lumina of the thyroid gland (ISHIKAWA, 1965; REMY et al., 1977; ISHIMURA and FUJITA, 1979; LUCIANO et al., 1979) or bile canaliculi in the liver (WOOD, 1965; DE WOLFPETERS et al., 1972). This is also the case when the smooth mucosa transforms to possess villi (MATHAN et al., 1976; MADARA et al., 1981; TOYOTA et al., 1989) or crypts (CHEN and KATAOKA, 1991) in developing small and large intestines, respectively. These previous authors suggested that the initial formation of an intraepithelial cavity includes the formation of a macula occludens between two epithelial cells and subsequent formation of an intraepithelial cavity surrounded by the two cells which are mutually joined by a zonula occludens, though the actual presence of such a cavity had not been evidenced by the freeze-fracture method until the present study. Some previous authors have shown intraepithelial cavities between two cells in thin sections, but the freeze-fracture method is the only way to demonstrate zonulae and maculae occludentes distinctly. This study was able to demonstrate precisely early events in intraepithelial cavity formation.

Intestinal epithelial cells are originally interconnected by the tight junction (zonula occludens) surrounding the intestinal lumen (primary or adluminal tight junction). It extends many open-ended tight junctional strands, basally. It is not clear whether the simplest type (Type I) of secondary tight junctions, i.e., free fragments of tight junctional strands, is formed de novo or of the open-ended strands being separated from the primary tight junction. The latter case seems economically more advantageous, by using the pre-existing attachment to make newly incorporated tight junctional proteins of the apposing plasmalemmas in register. For the same reason, a
secondary tight junction type II (macula occludens) must develop by adding new tight junctional proteins to the pre-existing type I tight junctional strands.

Intracellular cavities and small vesicles, similar to those observed in the present study, have been reported by many authors in hepatocytes (David, 1961; Wood, 1965; Yamamoto, 1965; Shin, 1978), in thyroid gland cells (Ishikawa, 1965; Shepard, 1968; Calvert and Pusterla, 1973; Remy et al., 1977; Yamashita et al., 1989) and in developing intestinal epithelium (Mathan et al., 1976; Madara et al., 1981; Toyota et al., 1989; Chen and Kataoka, 1991). Though it has been considered that the intracellular cavity and/or small vesicles open to the intercellular space between neighboring epithelial cells to form intercellular cavities or lumina, direct evidence that the first intercellular cavity forms within the secondary tight junction between two cells was only shown by Ishimura and Fujita (1979) in the developing thyroid gland and by the present study in the developing intestine.

Formation, enlargement and elongation of an intraepithelial cavity are explained by our present and previous studies as follows. The membrane of an intracellular cavity fuses with the plasmalemma within a macula occludens (secondary tight junction, Type II), the junctional strands of which are pushed laterally, and an intercellular (intraepithelial) cavity surrounded by a zonula occludens (secondary tight

Fig. 9. Various intracellular and intraepithelial cavities seen in the duodenal (D) and jejunal (A, B, C) epithelia on day 18. A. An intracellular cavity. ×28,000. B. Intracellular cavities (arrows) and small vesicles (arrowheads) open to an intracellular cavity (C). ×19,000. C. Small vesicles open to intermicrovillous regions and to sheath-like invaginations (arrows) surrounding microvilli. ×17,000. D. An intracellular cavity (arrow) opens to a branching intraepithelial cavity. ×12,000
junction, Type IV) eventually forms between two cells. The intraepithelial cavity is enlarged by the fusion of intracellular cavities and small vesicles (TOYOTA et al., 1989; CHEN and KATAOKA, 1990). Mitotic division of the cells results in the formation of a larger intraepithelial cavity surrounded by more than two cells which are mutually joined by a secondary tight junction, Type V. Junctional strands of secondary tight junctions tend to extend in various directions as shown in the present study. This tendency makes it possible for tight junctions enclosing neighboring intraepithelial cavities to fuse with each other followed by fusion of the intraepithelial cavities. Thus the intraepithelial cavity increasingly elongates to finally open into the main intestinal lumen. Such elongation of intraepithelial cavities together with an invasion of the mesenchymal core results in the formation of primitive villi, which are further divided to form definitive villi by a similar mechanism.

Morphological varieties of tight junctions shown in the present study, such as open-ended strands, tight junctions extending to apical plasmalemma, secondary tight junctions extending their strands toward various directions and cytoplasmic double-membraned vacuoles containing tight junctional strands, seem to represent the dynamic formation, extension and internalization of tight junctions during histogenesis.

**Gap junctions during villus formation and differentiation of absorptive cells**

The present study has shown for the first time that gap junctions develop and change their distribution in the intestinal epithelium during morphogenesis. In early stages, small gap junctions are associated with either primary or secondary tight junctions. As tight junctions mature, associated gap junctions become infrequent and disappear. On the other hand, gap junctions isolated from tight junctions appear and

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**Fig. 10.** Various gap junctions seen apart from tight junctions in duodenal (C) and jejunal (A, B, D, E) epithelia. x 300,000. A. A small gap junction consists of usual gap junctional particles and a few large particles (arrow) on day 14. B. A small gap junction consists of hexagonally arrayed large particles and a small number of usual gap junctional particles (arrowheads) on day 14. C. Hexagonally arrayed large particles (arrow) and usual gap junctional particles (arrowheads) form a medium-sized gap junction on day 18. D. A domain of scattered large particles (arrow) attaches to a large domain of usual gap junctional particles on day 20. E. A large gap junction accompanies a small number of solitary large particles (arrows) on day 17.
rapidly enlarge. These processes are fundamentally the same as those observed in the exocrine pancreas during fetal development (Yamamoto and Kataoka, 1985, 1988). Rapidly enlarging gap junctions accompany large particles in both tissues.

The study by Madara et al. (1981) was the only one to deal with gap junctions during morphogenesis of the rat intestinal mucosa. They observed the appearance and subsequent disappearance of small gap junctions associated with tight junctional strands, though they failed to notice the development of large gap junctions apart from tight junctions.

Gap junctions are considered to be sites of intercellular communication for maintaining the harmonious function of cells as well as sites for mediating cells' positional information for growth and differentiation (Wolpert, 1969; Loewenstein, 1979, 1981; Lo, 1980; Mehta et al., 1989). Though gap junctions develop by the same process in differentiating intestinal epithelium and pancreatic acini, mature cells of the two tissues exhibit an interesting contrast. In the pancreas, gap junctions develop rapidly during the proliferation and differentiation of exocrine cells and continue to be present in fully mature cells in the adult (Yamamoto and Kataoka, 1985, 1988). On the other hand, in the small intestine, large gap junctions are present at the site of epithelial cell proliferation and differentiation, i.e. intervillous regions in the developing mucosa (the present study) and intestinal crypts in the adult (Kataoka et al., 1989), but mature absorptive villous cells generally lack gap junctions. These facts suggest that gap junctions play an important role in cell proliferation and differentiation in both pancreatic exocrine and intestinal epithelial cells, but are required in the pancreas only for maintaining mature cell functions.

**REFERENCES**


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