Embryonic development of the myenteric nerve plexus in the rat small intestine as revealed by light and scanning electron microscopy

Hiroki Murata^{1,2}, Minoru Yagi², Masayuki Kubota², and Tatsuo Ushiki¹

¹*Division of Microscopic Anatomy and Bio-imaging, and* ²*Division of Pediatric Surgery, Niigata University Graduate School of Medical and Dental Sciences, Niigata, Japan*

Summary. This study was performed to clarify developmental changes in the structure of the myenteric nerve plexus in the rat small intestine. The small intestines of fetal and neonatal rats were examined by light microscopic immunohistochemistry for protein gene product 9.5 (PGP-9.5) and α -smooth muscle actin (α -SMA). Direct observation of the three-dimensional structure of the neuronal and muscular elements was accomplished by scanning electron microscopy of tissues after KOH treatment.

At the embryonic day 14 (E14), PGP-9.5 immunopositive (neuronal) cells first appeared in the mesenchymal tissue outside an α -SMA positive (muscle) cell layer. These cells increased in number to form a thin and continuous sheet, which was sandwiched between the inner and outer muscular layers at E17. This neuronal layer consisted of immature ganglion cells associated with supportive (enteroglia) cells, and was gradually segregated into groups by incomplete separation through slit-like interruptions at E18. With the widening of these slits at E19, the neuronal sheet was observed as a ladder composed of transversely elongated ganglia and their connecting nerve strands, resulting in the typical structure of the myenteric nerve plexus. Fibroblastic cells of a peculiar shape appeared on the outer surface of the myenteric plexus at E19. These cells (presumably interstitial cells) interconnected their long branching projections to form a cellular network on the myenteric plexus until birth.

Introduction

The enteric nerve system has two distinct nerve plexuses in the wall of the alimentary tract: the submucosal plexus of Meissner and myenteric plexus of Auerbach. The basic structure of these nerve plexuses has been studied by light microscopists using whole-mount preparations of the intestinal wall (Schabadasch, 1930; Stöhr, 1930; Irwin, 1931; Stach, 1971; Gabella, 1979, 1987; Kobayashi *et al.*, 1989; Wester *et al.*, 1998). These studies revealed that the myenteric nerve plexus is present between the inner circular and outer longitudinal muscular layers, and forms a lattice-work consisting of a number of ganglia with connecting nerve strands—although the size and shape of the meshes vary in different parts of the alimentary tract. Cellular components of the myenteric plexus were also studied extensively by light and electron microscopy which confirmed that ganglia of the plexus are composed of ganglion cells. The so-called "interstitial cells" are also known to form a reticular network on the ganglia (see review by Takayama *et al.*, 2002).

The three-dimensional organization of the enteric nerve plexuses was further studied by scanning electron microscopy (SEM). Fujiwara and Uehara (1980) were the first to succeed in directly visualizing the myenteric nerve plexus by SEM; they removed the extracellular matrix around the nerve plexus by treating the adult rat colon with a trypsin-HCl digestion method and observed the lattice-work of the myenteric plexus. Several other papers were subsequently published describing the shape of myenteric plexuses of different species by SEM (rabbit: Komuro, 1982; pig: Scheuermann *et al.*, 1986; guinea pig: Baluk and Gabella, 1987).

Embryologically, the enteric nervous system is derived from the cranial (vagal) and sacral neural crest (chick:

Received December 22, 2003

Address for correspondence: Dr. Hiroki Murata & Prof. Tatsuo Ushiki, Division of Microscopic Anatomy and Bio-imaging, Department of Cellular Function, Niigata University Graduate School of Medical and Dental Sciences, 1 Asahimachi-dori, Niigata 951-8510 Japan

Tel: +81-25-227-2062; Fax: +81-25-224-1767

E-mail: t-ushiki@med.niigata-u.ac.jp (Prof. T. Ushiki)

Hammond and Yntema, 1947; Yntema and Hammond, 1954; Andrew, 1969, 1970, 1971; mammalian: Webster, 1973; Rothmann and Gershon, 1982, 1984). Studies using various molecular markers have suggested that cranial neural crest cells first migrate into the proximal foregut of the embryonic day 9.0 (E9.0) in the mouse and spread to the caudal parts of the gut by E13.5 (Kapur *et al.*, 1992). Neurons were first detected morphologically in the foregut at E12 outside the inner muscular layer, increasing markedly between days E13 and E14 (Rothmann, 1982). Despite these previous studies on the development of the enteric nerves, the morphology of the enteric nervous system has not been fully investigated and, to our knowledge, no detailed study has been published on changes in the shape of the myenteric nerve plexus during prenatal development.

Thus, the aim of the present study is to reveal morphological changes in the myenteric nerve plexus during development more clearly and precisely than before. Since the presence of the lattice-work of the newborn myenteric plexus (Gabella, 1971, 1979; Schäfer *et al.*, 1999) implies that the basic formation of the plexus is completed in the prenatal period, we demonstrated the three-dimensional structure of the developing myenteric nerve plexus in the fetal rat small intestine by a combined use of light microscopy and SEM. The present study focuses on clarifying the first appearance of the neuronal elements in the rat intestine, lattice-work formation of the developing myenteric plexus, and differentiation of the interstitial cells in the fetal rat.

Materials and Methods

Animals

Embryonic and neonatal Wistar rats (SLC Inc. Hamamatsu) of both sexes were used in the present study. The embryonic day 0 (E0) was defined as the first day when the vaginal plaque was detected. Embryos were removed from the pregnant rats, which were killed by inhalation of ether followed by an intramuscular injection of an overdose of ketamine hydrochloride. Embryos at E13, 14, 17 and 18 were directly immersed in 4% buffered paraformaldehyde, while those at E20 were perfused from the left ventricle with 4% buffered paraformaldehyde and stored by immersion in the same fixative more than two days. Neonatal rats were anesthetized by being placed on ice and perfused transcardially with 4% buffered paraformaldehyde. The removed guts were stored in the same fixative at least for two days and prepared as follows.

Light microscopy

Whole embryos (at E13, 14 and 17) and the upper small intestine of neonatal rats were dehydrated in a graded series of ethanol and then embedded in paraffin. The specimens were cut into a series of horizontal sections at a 5 μ m thickness, and stained immunohistochemically with the anti-protein gene product (PGP 9.5) antibody (UltraClone Ltd., Isle of Wight, England) or the anti- α -smooth muscle actin (α -SMA) antibody (Sigma, St Louis, USA) as follows: The deparaffinized sections were treated with 0.1% trypsin (Wako, Osaka) for 15 min and fixed in the phosphate buffered saline (PBS) containing 0.3% Triton X-100 to increase the stainability. Absolute methanol containing 0.3% H₂O₂ was used for blocking the endogenous peroxidase activity. After a rinse in the PBS, the sections were incubated with normal goat serum (1:10; DAKO, Glostrup, Denmark) for 30 min and incubated overnight with the mouse anti- α -SMA antibody (1:800), or the rabbit anti-human PGP 9.5 antibody (1:250) at room temperature. They were then rinsed in PBS, and incubated either with peroxidase-conjugated goat anti-mouse immunoglobulins (1:100; P-0447, DAKO) for α -SMA, or peroxidase-conjugated goat anti-rabbit immunoglobulins (1:100; P-0448 DAKO) for PGP 9.5, for 2 h. Peroxidase activity was detected by incubating the sections with a 0.05M Tris-HCl buffer containing 3,3'-diaminobenzidine tetrahydrochloride (DAB: Wako) and 0.003% H₂O₂. The immunostained sections were counterstained with hematoxylin.

A part of the small intestine of the neonatal rat after fixation was directly stained immunohistochemically with the anti-PGP 9.5 antibody *in toto* and observed as a whole mount preparation.

Scanning electron microscopy

The fixed small intestines of fetal and neonatal rats were further immersed in a 2% glutaraldehyde in 0.1M phosphate buffer (pH7.4) for more than 2 days at 4°C. In order to remove the extracellular matrix selectively, we used a KOH digestion method (Ushiki and Murakumo, 1991; Yamada and Ushiki, 2001); namely, the fixed specimens were immersed in 30% KOH for 8 min at 60°C. After being rinsed in a 0.1M phosphate buffer several times, they were conductive-stained by treatment with 1% tannic acid for 3 h, a phosphate buffer for 1 h, and 1% OsO₄ for 3 h at room temperature. The tissues were dehydrated in a graded ethanol series, transferred to isoamyl acetate, and dried in a critical-point dryer with liquid CO₂. The dried specimens were coated with platinum-palladium in an ion coater and observed in an SEM (S-2380N, Hitachi, Tokyo) at an accelerating voltage of 10 kV.

Fig. 1. Cross-sectional views of the rat small intestine at E13 (a, b, c, d) and E14 (e, f, g). The sections for microscopy were either stained with hematoxylin and eosin (a, e) or immunostained with the α -SMA antibody (b, f) or PGP-9.5 antibody (c, g). **a–c:** At E13, the mesenchymal tissue is rather uniform in structure and no immunoreactivity for α -SMA (b) or PGP-9.5 (c) is detected in the intestinal wall. **d:** The mesenchymal tissue layer is observed as an aggregation of irregularly shaped cells by scanning electron microscopy. **e–g:** At E14, the mesenchymal tissue is subdivided into three layers: inner pale, middle dense and outer pale layers at E14. α -SMA immunopositive cells (f) are found in the upper half of the middle dense layer, while PGP-9.5 immunopositive cells (g) form small groups in the lower half of the middle layer. e: epithelium. Scale bars: 50 μ m

Fig. 2. The rat small intestine at E17. **a:** In a cross section of the intestinal wall, PGP-9.5 immunopositive cells are found between the inner (im) and outer (om) muscular layers. **e:** epithelium. **b** and **c:** Scanning electron micrographs showing an overview of the neuronal layer (i.e., immature myenteric plexus) after removal of the outer muscular layer. Many spherical neuronal cells and their fibers are observed in **a**, while the neuronal layer consists mainly of irregularly shaped cells in **b**. An arrow indicates a nerve strand in the neuronal layer. **d:** A closer view of the boxed area in **c** shows that the nerve strand is a bundle of axons partially enclosed by a thin process of the irregular shaped (glial) cell. om: outer longitudinal muscular layer. Scale bars: 50 μ m (a, b, c), 10 μ m (d)

Fig. 3. The outer surface of the myenteric plexus (colored yellow) of the rat small intestine at E18. The outer muscular layer (om) has been partially removed in this specimen. Slit-like interruptions (arrows) are observed in places in the neuronal sheet. The surface of the neuronal sheet is roughly covered with irregularly shaped flattened cells. Several spherical cells or ganglion cells (n) are also found in the portion where the superficial flattened cells (or immature enteroglia cells) have been artificially partially removed during preparation. Note the inner muscular fibers (im) beneath the neuronal layer of the myenteric plexus. Scale bar: 50 μ m

Fig. 3. Legend on the opposite page.

Fig. 4. The outer surface of the myenteric nerve plexus (yellow) at E19. In the portion where the outer muscular layer (om) has been removed, the nerve plexus is observed as elongated masses arranged roughly parallel in a circular fashion. These masses, or ganglia, are connected with each other through thin nerve strands (arrows), resulting in the formation of the lattice-work in the myenteric nerve plexus. Inner circular muscle cells (im) are found between the ganglia. Scale bar: 50 μ m

Results

Embryonic day 13 and 14 (E13–14)

By light microscopy, the wall of the upper small intestine in the early embryonic stage consisted mainly of the mesenchymal tissue sandwiched between the midgut epithelium

and thin mesothelium. At E13, the mesenchymal tissue was rather uniform in structure, and no immunoreactivity for α -SMA nor PGP-9.5 was detected in the intestinal wall (Fig. 1a–c). By SEM, the mesenchymal tissue layer was observed as an aggregation of irregularly shaped cells (Fig. 1d).

At E14, the mesenchymal tissue was subdivided into

Fig. 5. Closer view of the outer surface of the myenteric plexus at E19. Ganglia are covered with flattened immature glial cells (g). Note that connecting nerve strands (arrows) are composed of a bundle of naked axons. im: inner muscular layer. Scale bar: 20 μ m

three layers: inner pale, middle dense, and outer pale. Among these, α -SMA immunopositive cells were found in the middle dense layer although they tended to accumulate predominantly in the upper half of this layer; these cells were considered immature smooth muscle cells in nature (Fig. 1f). On the other hand, PGP-9.5 immunopositive cells formed small groups in the lower half of the middle layer in the mesenchymal tissue (Fig. 1g).

Embryonic day 17 (E17)

The mesenchymal tissue of the intestinal wall was characterized by the presence of a thickened pale layer surrounded by a thin smooth muscle layer and mesothelium. PGP-9.5 immunopositive cells formed a roughly continuous sheet in the middle of the muscle layer, resulting in the subdivision of the muscle layer into the thick inner and thin

Fig. 6. The outer surface of the myenteric plexus at E19. **a:** In portions where glial cells are removed from the ganglia, the spherical ganglion cells (n) are exposed. These ganglion cells appear partially embedded in the inner circular layer (im). g: ganglion. **b:** The ganglion cells in the ganglia are directly observed probably because the superficial glial cells have been artificially removed during the sample preparation. **c:** In this specimen, almost all ganglion cells (n) have been removed artificially. Transverse depressions on the surface of the inner muscular layer correspond to portions where ganglion cells were accommodated. Scale bars: 50 μ m

Fig. 7. The outer surface of the myenteric plexus at E19 (a) and E20 (b). **a:** Fibroblastic cells (immature interstitial cells, colored blue) are found on the myenteric plexus. These cells are spindle in shape and arranged longitudinally along the intestinal tube, attaching to the surface of the ganglia. **b:** At E20, the fibroblastic cells (blue) on the outer surface of ganglia are stellate with several branched projections, and form a coarse network by connecting their projections with each other. Scale bars: 50 μm

outer layers (Fig. 2a). Thus, the PGP-9.5 immunopositive cell layer was considered to form the immature myenteric nerve plexus.

By SEM, the layer of the immature myenteric nerve plexus was observed in portions where the outer muscular layer was accidentally removed during preparations. This layer consisted of spherical and/or irregularly shaped cells which were arranged in a continuous sheet like a pavement; spherical neuronal cells were apparently dominant in some places (Fig. 2b), while irregularly shaped cells occupied other sites (Fig. 2c). Axon bundles and independent axons were often observed in this layer although their arrangement was rather random. At high magnification, axon bundles were sometimes partially covered by processes of irregularly shaped cells (Fig. 2d), suggesting that these were glial cells in nature.

Embryonic day 18 (E18)

Although the myenteric nerve plexus formed a continuous sheet, small interruptions appeared in places in it. These

partial interruptions were observed as slender slits running circularly to the axis of the intestine. At higher magnification, the surface of the nerve plexus appeared to be roughly covered with flattened cells of irregular shapes.

In portions where the superficial flattened cells of the nerve plexus were artificially and partially removed, the spherical neuronal cells were exposed to the surface, indicating that a group of immature ganglion cells was unwrapped by a thin sheet of glial cells at this stage (Fig. 3).

Embryonic day 19–20 (E19–20)

The myenteric nerve plexus was observed as a number of elongated masses which were arranged roughly parallel in a circular fashion. These elongated neuronal masses apparently correspond to ganglia of the myenteric plexus. Individual ganglia branched or fused with each other along their course, while they interconnected through thin nerve strands. At high magnification, some of these strands appeared as a bundle of naked axons, while the others were

Fig. 8. Legend on the opposite page.

axon bundles associated with a flat process of the glial cells (Fig. 4, 5). In one place where almost all neuronal elements of the ganglia were accidentally removed during the sample preparation, transverse depressions were marked on the surface of the inner circular muscle layer, indicating that each ganglion was partially embedded in the inner circular layer (Fig. 6a, b, c).

Fibroblastic cells, probably corresponding to immature interstitial cells, appeared at E19 on the outer surface of the myenteric plexus. These cells were spindle-shaped and apparently attached to the surface of the ganglia, being arranged roughly longitudinally along the intestinal canal (Fig. 7a). At E20, these cells had several branched projections, and formed a coarse network by interconnecting their projections (Fig. 7b).

Newborn

Immunohistochemically, the PGP-9.5 positive myenteric ganglia were obvious between the inner and outer muscular layers. In addition to this plexus, the submucosal plexus of Meissner appeared after birth. Connections between the submucosal and myenteric plexuses were also observed (Fig. 8a). In a whole mount specimen, the myenteric plexus was observed like a ladder due to the presence of circularly arranged elongated ganglia and their connecting strands (Fig. 8b). In SEM observations, the distance between the two neighboring ganglia was wider than that in the fetal period (Fig. 8c). At high magnification, the glial cells were observed as flattened cells enwrapping the group of ganglion cells and bundles of nerve fibers (Fig. 9).

The fibroblastic (interstitial) cells were well developed on the outer surface of the myenteric plexus; they extended highly branched projections to form an elaborate cellular network which surrounded the ganglia like a basket (Fig. 10). Flattened fibroblasts were also found on the surface of the myenteric ganglia.

Discussion

The present study has revealed the structure of developing myenteric nerve plexus by light microscopy and SEM. We have clarified the following three points of note: 1) the appearance of neuronal elements in the rat intestinal wall, 2) changes in the structure of the developing myenteric plexus in the fetal period, and 3) the development of interstitial cells. In particular, the present study is the first to demonstrate the precise structure of the individual cellular components (i.e., immature ganglion cells, enteroglia cells and interstitial cells) three-dimensionally in the developing myenteric plexus of fetal rats by SEM. Previously, several digestion methods for removing collagenous components were used with intestinal tissues for directly visualizing the myenteric plexus by SEM (Fujiwara and Uehara, 1980; Komuro, 1982; Scheuermann *et al.*, 1986; Baluk and Gabella, 1987) because the plexus is hidden in surrounding connective tissues. In the present study, we succeeded in exposing the myenteric nerve plexus in the fetal rat by a KOH digestion method. This KOH digestion method has been applied by our research group to three-dimensional observations of various tissues and organs (e.g., Ushiki and Murakumo, 1991; Ushiki and Watanabe, 1997) including fetal materials (Iino *et al.*, 2001; Yamada *et al.*, 2002). The present study has further confirmed that this method is suitable for visualizing the structure of the rat enteric plexus without any severe deformation of the cellular components, even in the fetal tissues and organs.

First appearance of the neuronal elements

In the present study, we have demonstrated that PGP-9.5 positive (neuronal) cells appear at E14 outside the α -SMA positive cells in the rat small intestine. Although there is a possibility that the PGP-9.5 antibody does not react to the most immature neuronal cells, our findings indicate that the enteric nerve plexus develops at least until E14 in the rat. Previous studies revealed that the neurons derived mainly

Fig. 8. The myenteric plexus at birth. **a:** A cross section of the small intestine immunostained with the PGP-9.5 antibody. The immunoreactive myenteric ganglia (of Auerbach; arrows) are present between inner (im) and outer (om) muscular layers. The submucosal plexus (of Meissner; double arrows) are also clearly displayed in this specimen. Note a connection (arrowhead) between the submucosal and myenteric plexuses. **b:** A whole mount specimen immunostained with the PGP-9.5 antibody. The myenteric plexus is observed like a latticework due to the presence of circularly arranged ganglia and their connecting strands. **c:** An SEM image showing the outer surface of the myenteric nerve plexus (colored yellow). The plexus form a latticework with ganglia and nerve strands, though the distance between the two neighboring two ganglia is wider than that in the earlier stage. A network of fibroblastic cells (colored blue) has developed on the outer surface of the myenteric plexus. Scale bars: 50 μ m (a), 100 μ m (b), 20 μ m (c)

Fig. 9. A closer view of the myenteric nerve plexus at birth. Ganglion cells (n) are directly observed, probably because glial cells (g) enfolding the ganglion cells and axons (arrow) were partially removed during the sample preparation. Note the sheet of flattened glial cells in the lower left of this micrograph. im: inner muscular layer. Scale bar: 20 μ m

Fig. 10. The outer surface of the myenteric plexus at birth. The fibroblastic cells, or interstitial cells (colored blue), are well developed to form a cellular network surrounding the ganglia. Note flattened fibroblasts (colored green). im: inter muscular layer, om: outer muscular layer. Scale bar: 50 μ m

from precursor cells originally located in the vagal or lumbosacral segment of the neural crest that migrate into cranial portion of the avian and mammalian gut (Andrew, 1971; Le Douarin and Teillet, 1973; Webster, 1973; Rothmann and Gershon, 1984). A histochemical study for acetylcholinesterase also showed that the rat vagal nerves spread over the stomach and beyond the pylorus into the duodenal loop at E14 (Cantino, 1970). Thus, the neural crest cells probably migrate into the intestinal wall around E14.

The present study has also demonstrated that the neu-

ronal elements which first appear in the intestinal wall are the ganglia of the myenteric plexus; the submucosal plexus appears after birth. Several investigators have already pointed out the migration of neurons from the myenteric plexus into the submucosa to form the submucosal plexus in various species (Okamoto and Ueda, 1967; Gershon *et al.*, 1981; Pham *et al.*, 1991; McKeown *et al.*, 2001).

The development of non-neuronal supportive cells or enteroglial cells in the enteric nerve system has been a matter of dispute. Gershon *et al.* (1981) observed the fetal

guinea-pig intestine by transmission electron microscopy and described presumptive ganglion cells and supportive cells in the intestinal wall at E25. On the other hand, in their immunohistochemical studies on the rat colon and rectum, Nada and Kawana (1988) reported that the immunoreactivity to glial fibrillary acidic (GFA) protein and S-100 protein in the gut were only found in the postnatal period. The present study has clearly revealed that the enteroglial cells appear at a rather earlier stage (i.e., at least by E17 in the rat). Our unpublished data show that the immunoreactivity of anti S-100 protein cannot in fact be detected in the prenatal stage of the rat, probably due to the immaturity of enteroglial cells in the fetal period, in contrast with mature enteroglial cells with their strong S-100 immunoreactivity (Kobayashi *et al.*, 1986).

Development of the ladder formation in the myenteric plexus

The meshwork of the myenteric nerve plexus has been reported in whole-mount specimens in various species such as the chick (Keller, 1976), guinea-pig (Gabella, 1987), rat (Nada and Kawana, 1988; Schäfer *et al.*, 1999), and dog (Ward and Torihashi, 1995) although the shape and size of the meshwork differ by region and species. The shape of the myenteric plexus in the adult rat small intestine is like a ladder or lattice, which consists of elongated ganglia and their connecting nerve strands; the ganglia are arranged transversely to the intestinal axis, while the connecting nerve strands run in a longitudinal direction. The present study has been first to clarify the process of lattice-work formation in the myenteric plexus. As shown in previous studies, the lattice-work of the myenteric nerve plexus is already formed at birth although several researchers noted that each ganglion is observed to be much closer in newborn than in adult animals (Gabella, 1979; Schäfer *et al.*, 1999). In the present study, we have clearly demonstrated that the latticework of the myenteric plexus is produced by the formation and enlargement of slits in the continuous neuronal sheet in the fetal intestine.

It is interesting that enteroglial cells are already present when the slits are produced in the neuronal sheet. Our SEM findings have clearly shown that a group of the ganglion cells are roughly enwrapped by flattened enteroglial cells, even in this period. This indicates that the slits are made by the segregation of ganglion cells with enteroglial cell sheaths.

Another noteworthy finding is that axon bundles corresponding to the connecting nerve strands were found in the early stage of plexus formation. In our SEM studies, the immature enteroglial cells wrap the axon bundles even at the early fetal stages (i.e., E17), suggesting that the

enteroglial cells may be involved in the axon bundle (or nerve strand) formation of the plexus.

Interstitial cells

The presence of fibroblastic cells associated with the myenteric nerve plexus has been reported by various investigators using light microscopy (Taxi, 1952; Burns *et al.*, 1997) and TEM (Thuneberg, 1982; Fausone-Pellegrini, 1985, 1987) and SEM (Baluk and Gabella, 1987; Kobayashi *et al.*, 1989; Komuro, 1989; Komuro *et al.*, 1999). These cells have a spindle or globular shape with slender branched processes to form a meshwork covering the myenteric plexus. These cells are currently referred as interstitial cells—although the terminology is somewhat confusing even at present (see review by Kobayashi, 1990, 1996; Komuro *et al.*, 1999; Sanders *et al.*, 1999; Takayama *et al.*, 2002). Since the interstitial cells are intercalated between the intramural neurons and smooth muscle cells, they are believed to form a gastroenteric pacemaker system by generating electrically slow waves. It is thus important to analyze the structural details of the development of interstitial cells for understanding the function of the developing intestine. The present study provides several new findings on this matter as it is the first to demonstrate three-dimensionally the structure of the developing interstitial cells in the fetal rat intestine.

In their TEM studies of the mouse intestine, Fausone-Pellegrini *et al.* (1985, 1996) reported that interstitial cells and their precursor cells were not identifiable before birth. On the other hand, Torihashi *et al.* (1997) described c-kit positive interstitial cells as being first detected in the outer layers of the gut of E12.5 mice. In the present study, we have shown that the interstitial cells in the rat intestine appear by E19, independently of the ganglion cells and enteroglial cells.

Several investigators proposed that interstitial cells in the myenteric plexus are derived from bipotential progenitors that are able to develop along the interstitial cells or smooth muscle cells (Klüppel *et al.*, 1998; Young, 1999). The longitudinal arrangement of the immature interstitial cells on the outer surface of the myenteric plexus may indicate that these cells are related to immature outer muscle layers. Studies in the combined use of SEM and other techniques such as c-kit expression will provide further information on the structure and function of the interstitial cells during development.

Acknowledgements

We thank the staff of Division of Microscopic Anatomy and Bio-imaging, Niigata University Graduate School of

Medical and Dental Sciences, for their generous support throughout this study. Thanks are especially due to Mr. Masaei Takeda for his technical assistance.

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