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REGULAR ARTICLE

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Ultrastructural characterization of interstitial cells of Cajal in the rat small intestine using control and *Ws/Ws* mutant rats

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Abstract Interstitial cells in the myenteric plexus and the deep muscular plexus of the small intestine of the c-*kit* mutant rats (*Ws/Ws*) and their normal siblings (+/+) were studied. c-Kit immunoreactivity was detected in two regions corresponding to the myenteric plexus and the deep muscular plexus in the jejunum of +/+ rats, while no immunoreactivity was detected in *Ws/Ws* rats. Using electron microscopy, two types of gap junction-forming interstitial cells were found in association with the myenteric plexus in +/+ rats: one type characterized by a typical fibroblastic ultrastructure, and the other characterized by numerous mitochondria and less electron-dense cytoplasm. Since the latter were greatly reduced in *Ws/Ws* rats, it was suggested that these cells correspond to c-*kit*-expressing cells, i.e. interstitial cells of Cajal in the myenteric plexus region. In contrast, two types of interstitial cells in the region of the deep muscular plexus were observed with no difference between +/+ and *Ws/Ws* rats. Probable interstitial cells of Cajal in this region were characterized by a basal lamina and numerous caveolae as well as large gap junctions that interconnect with each other and with the smooth muscle cells. We concluded that interstitial cells of Cajal in the rat intestine are heterogeneous in ultrastructure, c-*kit* dependency in the cell maturation, and functional role.

Key words Interstitial cells of Cajal (ICC) · c-Kit · Small intestine pacemaker · Morphology · Rat (*Ws/Ws*)

Introduction

Recent studies indicate that the cells expressing c-Kit receptor in the mouse small intestine correspond to interstitial cells of Cajal (ICC; Cajal 1911), which have been proposed as the pacemakers of intestinal peristalsis (Thuneberg 1982), by demonstrating that the postnatal blockade of the receptor with its antibody (Maeda et al. 1992; Torihashi et al. 1995) or its genetic defect (Ward et al. 1994; Huizinga et al. 1995) results in a loss of pacemaker activity or electrical slow waves in the intestine. Abnormalities in the ileal movement and pyloric sphincter function have also been reported in *Ws/Ws* rats in which the tyrosine kinase activity of c-Kit is severly impaired (Isozaki et al. 1995). The gene product of c-*kit* is a receptor tyrosine kinase and is encoded by the mouse *W* locus and the rat *Ws* locus (Chabot et al. 1988; Tsujimura et al. 1991). The extracellular domain contains the receptor for stem cell factor (SCF), the natural ligands for c-Kit receptors, and the cytoplasmic domain conveys tyrosine kinase activity. SCF is encoded by the mouse *Sl* locus (Williams et al. 1990; Zsebo et al. 1990).

Isozaki et al. (1995) have reported that a small number of c-*kit* expressing cells are present in the small intestine in contrast to the complete absence of those cells in the stomach of *Ws/Ws* rats, and have suggested that the development of ICC in the intestine could be less dependent on the c-Kit-SCF system than their development in the stomach. They have also suggested the possibility of the presence of c-*kit*-negative ICC in the gastrointestinal tract.

A most interesting question is whether different classes of ICC constitute morphological and functional subtypes of ICC or represent different cell lineages, since Thuneberg (1982) has classified four types of ICC for the first time based on their tissue locations and ultrastructural features. Recently, Burns et al. (1997) classified six types of ICC in the guinea-pig gastrointestinal tract by using c-Kit immunohistochemistry.

The present study intends to characterize the ultrastructural features of ICC in the deep muscular plexus (ICC-DMP) and Auerbach's (myenteric) plexus (ICC-AP), corresponding to c-*kit* expressing cells in each location by a comparison of *Ws/Ws* rats and their normal siblings. Although similar studies using either W/W^v mouse, which has a point mutation of c-*kit* (Malysz et al. 1996), or Sl/Sl^d mouse, which has a mutation in the ligand for c-Kit (Ward et al. 1995), have reported differences between

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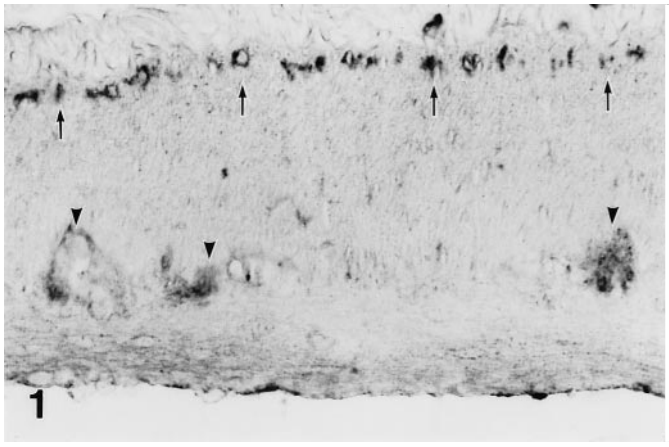


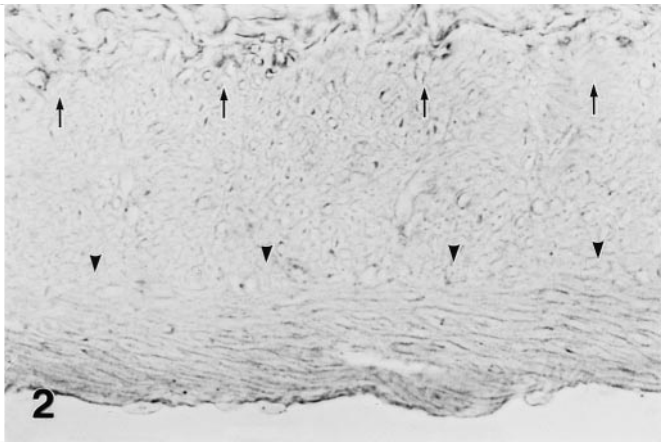


Fig. 1 Dense immunoreactive deposits to anti-c-Kit antibody are observed in the region corresponding to the DMP (*arrows*) in the control +/+ rat small intestine. Moderate immunoreactivity is also seen in the region of the myenteric plexus (*arrowheads*). ×320

Fig. 2 No immunoreactivity to anti-c-Kit antibody is observed in the regions corresponding to the DMP (*arrows*) and to the myenteric plexus (*arrowheads*) in *Ws/Ws* rat small intestine. ×320

ICC-DMP and ICC-AP on c-Kit dependency in cell maturation, their ultrastructures have not been thoroughly examined. Besides, it has been known that the DMP region of both rats (Komuro and Seki 1995; Seki and Komuro 1997) and guinea-pigs (Zhou and Komuro 1992a,b) and the myenteric region of guinea-pigs (Komuro et al. 1996) contain more than one type of interstitial cells forming close contacts with nerve terminals and gap junctions with smooth muscles which are believed to be characteristic features of ICC. Thus, a critical examination of those cells seems to be necessary to further clarify the cytological nature of the non-neural regulatory system, including ICC, in the gut motility. A preliminary account of this study has been published elsewhere (Horiguchi et al. 1995).

Materials and methods

Immunohistochemistry

Seven homozygous *Ws/Ws* mutant rats and sibling control +/+ rats (aged 4–8 weeks) were used. Under terminal anesthesia with ether, short segments of proximal jejunum were removed from these animals and frozen in liquid nitrogen in OCT compound (Tissue Tek). Cryostat sections were cut at a thickness of 10 μm and collected on gelatin-coated slides. The specimens were fixed with acetone for 10 min at room temperature, rinsed in phosphate-buffered saline (PBS) several times, and incubated with 4% Block Ace solution (Dainippon Seiyaku) for 20 min at room temperature to prevent nonspecific antibody binding. Then specimens were incubated overnight at 4°C with a primary antiserum against human c-Kit protein (C-19; SantaCruz Biotechnology, rabbit polyclonal), at a dilution ratio of 1:50 in PBS-azide. After washing in PBS, the specimens were further incubated overnight at 4°C with peroxidase-conjugated secondary antibodies (DAKO, swine anti-rabbit IgG) at a dilution ratio of 1:50. Horseradish peroxidase reaction was developed in 50 ml 0.1 M TRIS-HCl buffer (pH 7.4) solution containing 6 mg 4-chloro-1-naphtol (Sigma) and 8 μg 30% H_2O_2.

Electron microscopy

Short segments of proximal jejunum were removed with the animals under ether anesthesia, and placed in a fixative containing 3% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer, pH 7.2, for 2 h at 4°C. The specimens were rinsed in the same buffer and post-fixed in 1% osmium tetroxide for 2 h at 4°C. The specimens were then rinsed in distilled water, block-stained with saturated uranyl acetate solution for 3 h, dehydrated in a graded series of ethyl alcohols and embedded in Epon epoxy resin. Ultrathin sections were cut using a Reichert microtome and double-stained with uranyl acetate and lead tartrate for observation under a JEM 1200EX II electron microscope.

For counting the cell number of interstitial cells in the myenteric region, only cell profiles with a nucleus were counted along the border of the circular muscle cells on montages of electron micrographs of the control +/+ and *Ws/Ws* rats.

Results

c-Kit immunohistochemistry

Immunoreactivity to anti-c-Kit antibody was clearly observed in two regions corresponding to the DMP and the myenteric plexus in the jejunum of +/+ control rats (Fig. 1). In contrast to the control rats, no immunoreactivity was detectable in the myenteric plexus region or the DMP region of *Ws/Ws* rats (Fig. 2).

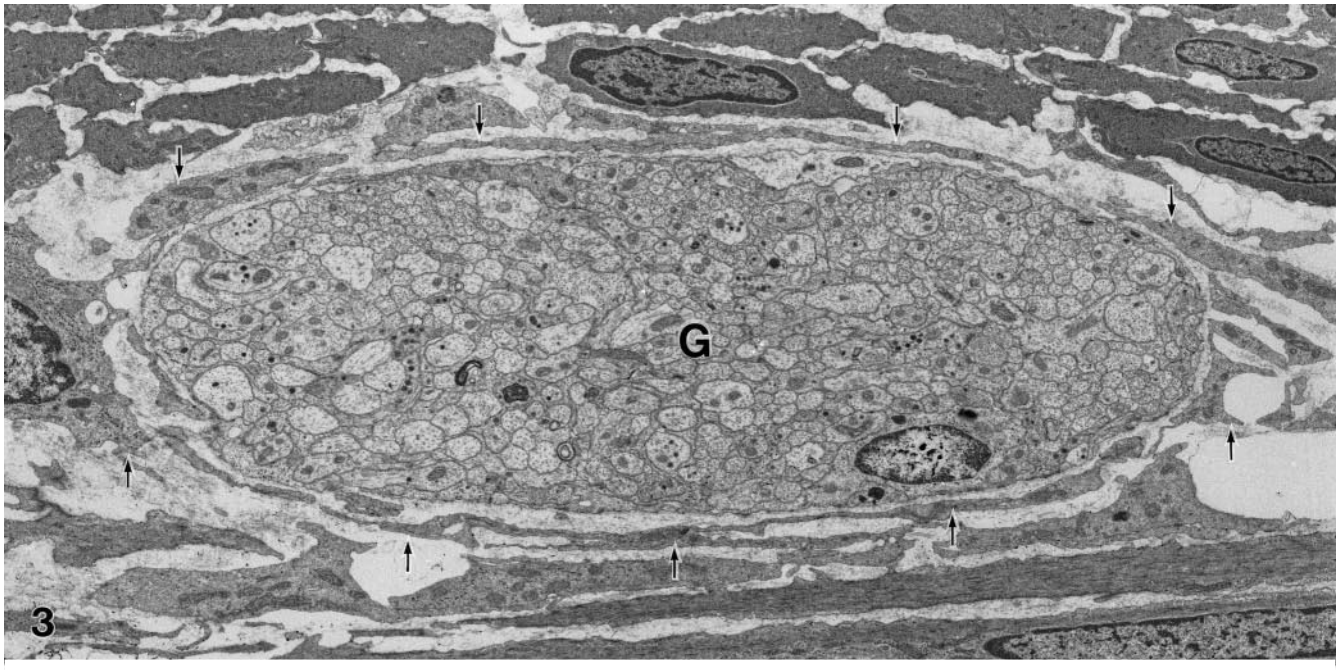
Interstitial cells in the myenteric region

Control +/+ rats. Many interstitial cells are observed in this region so that the myenteric ganglia are usually sur-

Fig. 3 Electron micrograph showing a myenteric ganglion (*G*) surrounded by a nearly complete sheath consisting of interstitial cells and their processes (*arrows*) in the control +/+ rat small intestine. ×7000

Fig. 4 A fibroblast-like cell located in the myenteric plexus region in the control +/+ rat. Well-developed RER is conspicuous and its cisterns contain moderate electron-dense material. It forms a small gap junction with smooth muscle cells (*arrow*). ×20000. *Inset* Higher magnification of the gap junction indicated by the *arrow*. ×80000

Fig. 5 ICC-AP observed in +/+ rat, which is characterized by many mitochondria and less electron-dense cytoplasm. ×12000. *Inset* A gap junction between slender cytoplasmic processes of ICC-AP. Note, there is no basal lamina around the cell membrane. ×96000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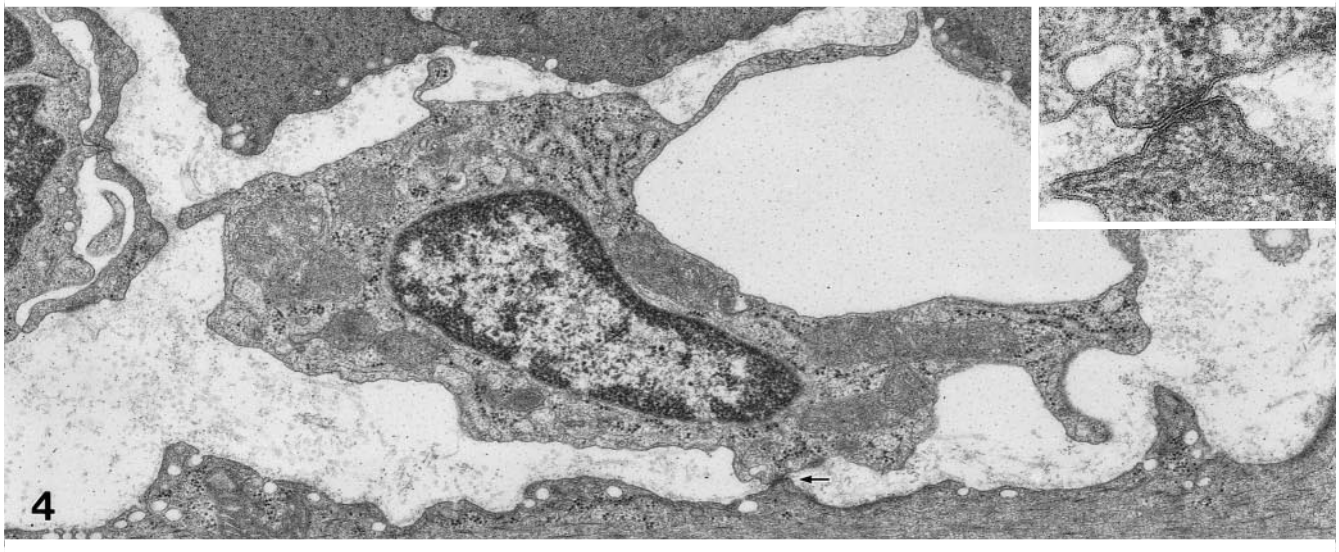
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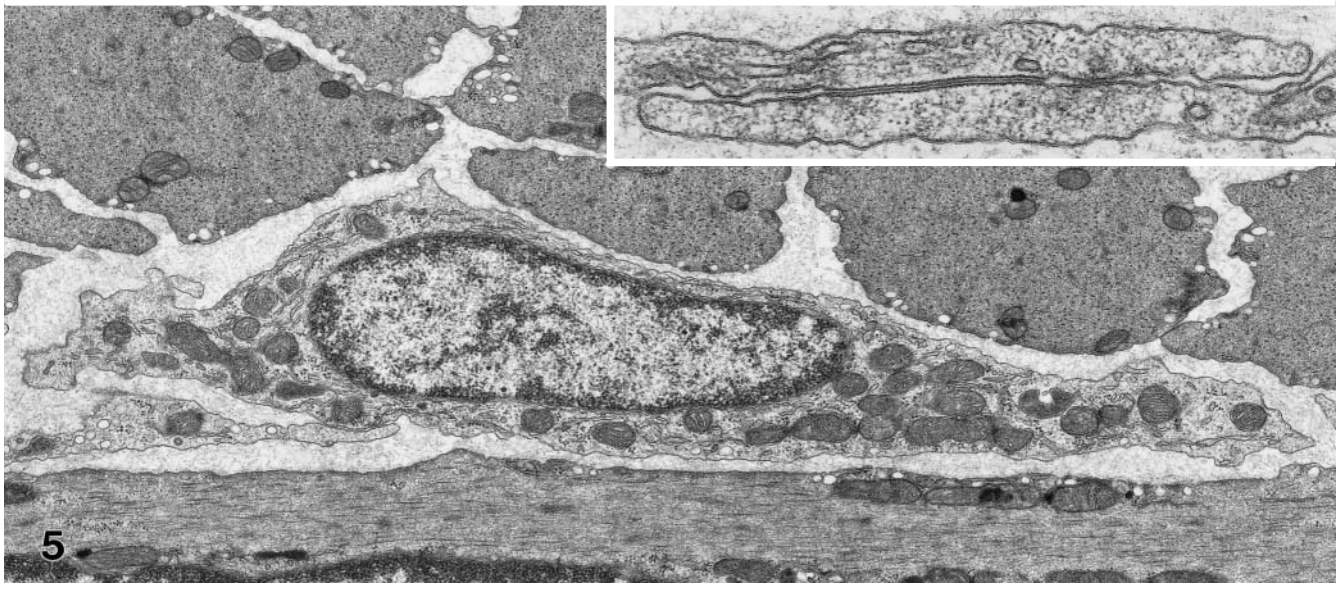
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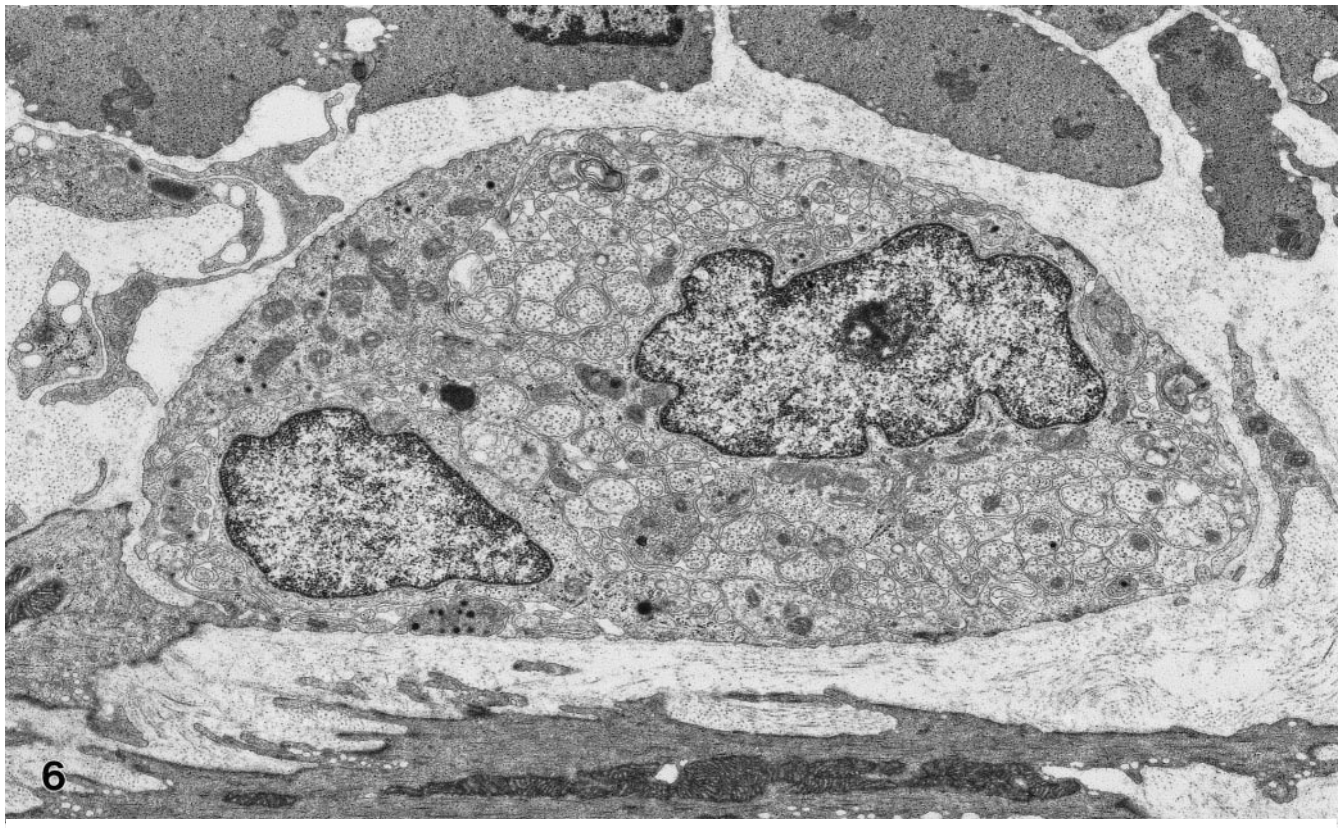


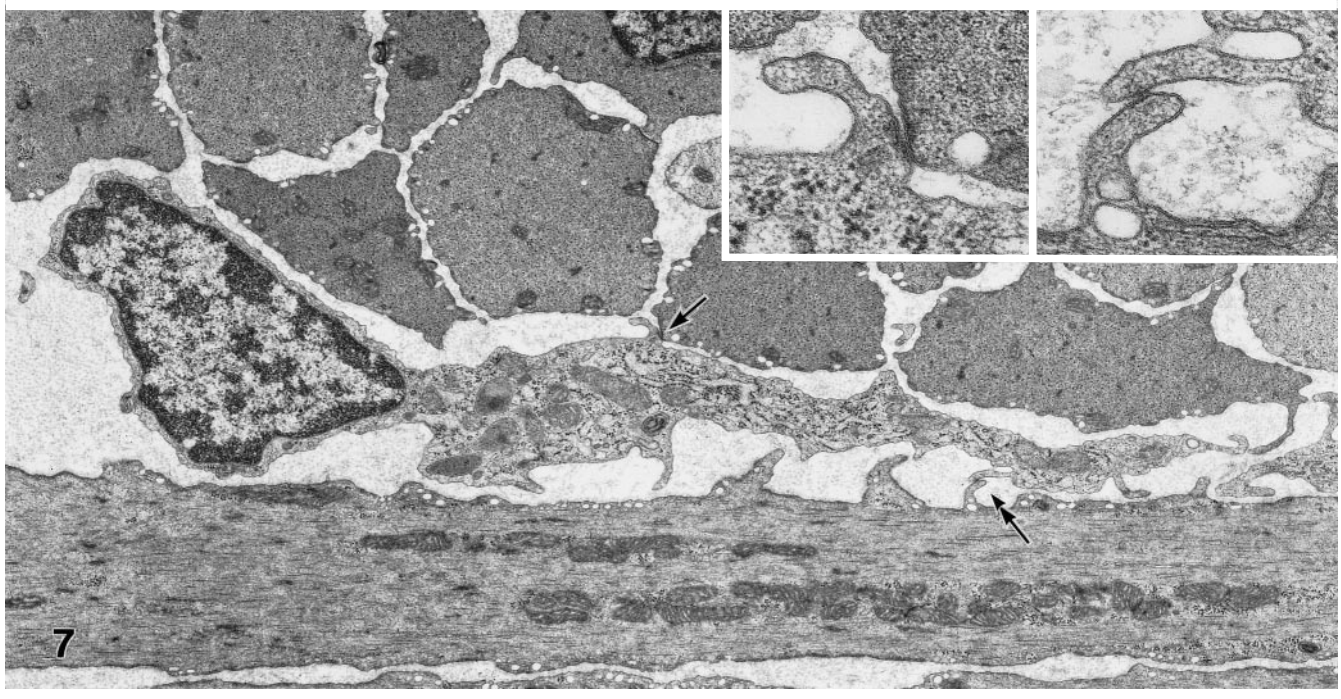
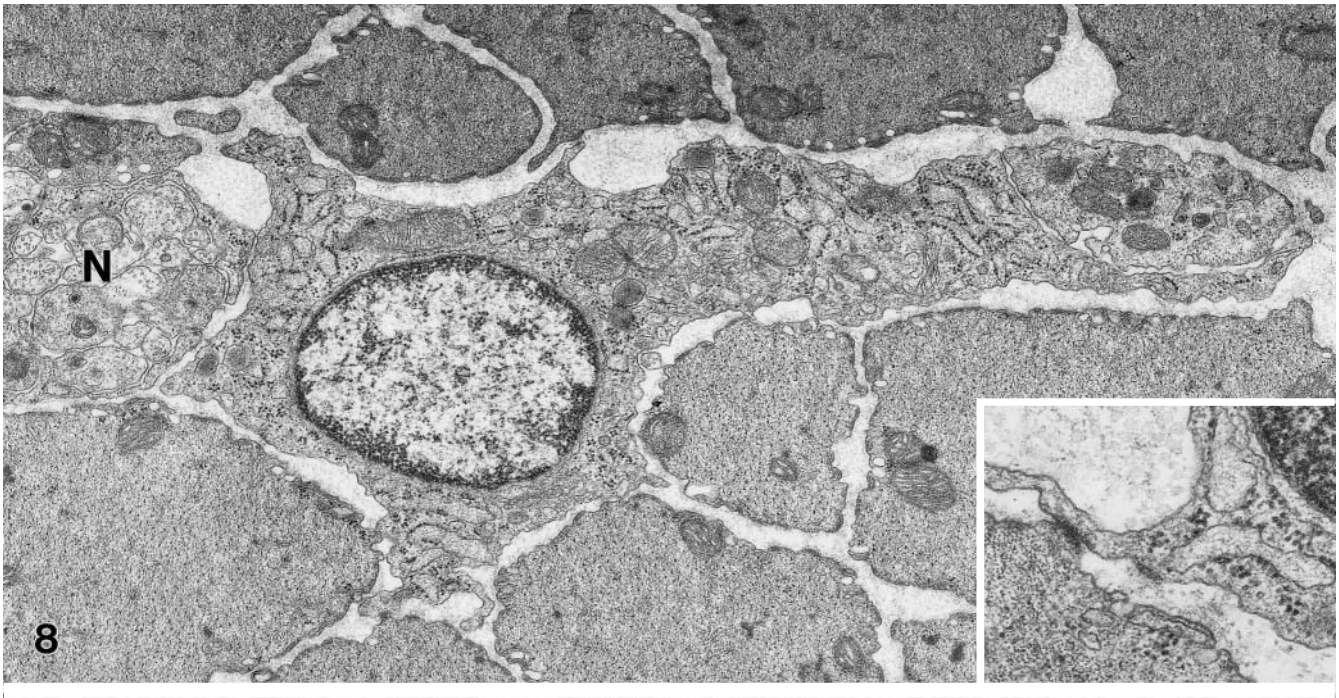


Fig. 6 A myenteric ganglion in *Ws/Ws* rat, which is accompanied by a few processes of interstitial cells. ×11000

Fig. 7 A fibroblast-like cell located in the myenteric region of *Ws/Ws* rat. It forms small gap junctions with the muscle cells of both circular (*arrow*) and longitudinal (*double-headed arrow*) layers. ×10000. *Inset* Higher magnification of the gap junction indicated by an *arrow* and a *double-headed arrow*. ×52000

rounded by an almost continuous sheet of their cytoplasmic processes(Fig. 3). Two types of the interstitial cells were distinguished by their ultrastructural features.

Cells of the first type show features of typical fibroblasts (Fig. 4). Their nuclei are elongated and have condensed chromatin. They have well-developed rough endoplasmic reticulum (RER) of which cisterns are often dilated, and contain moderately dense material. Free ribo-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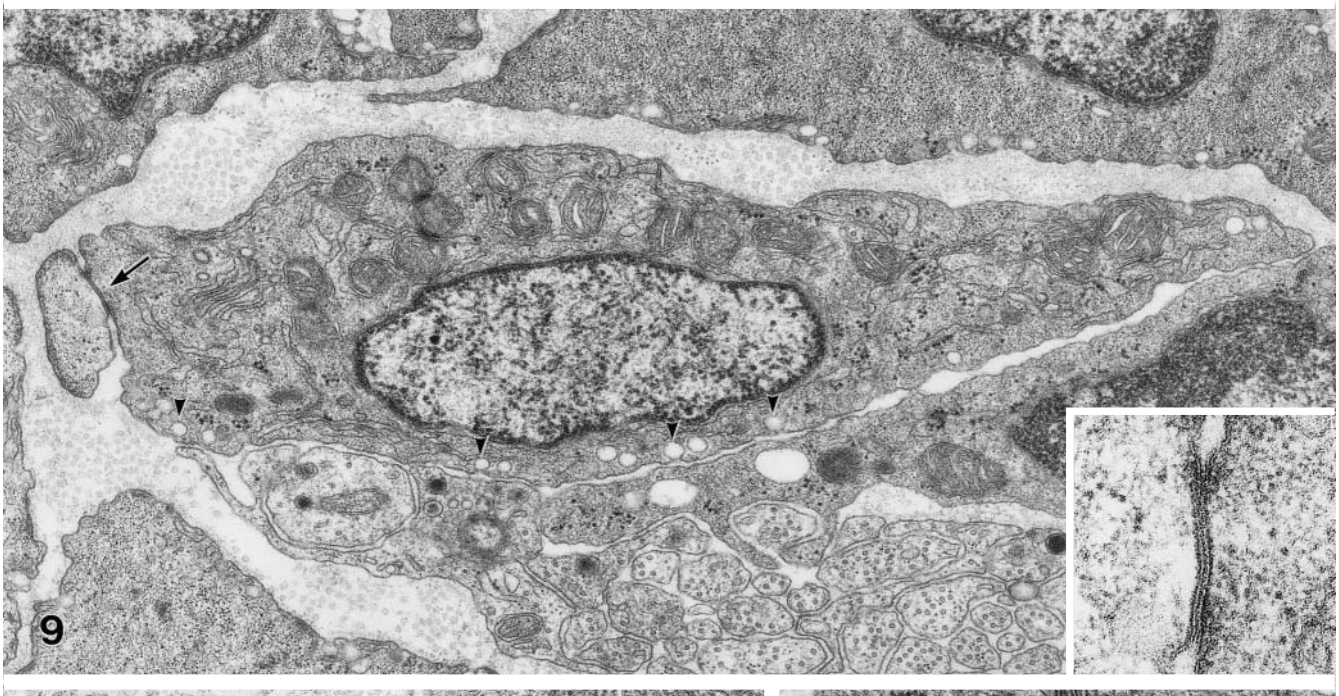
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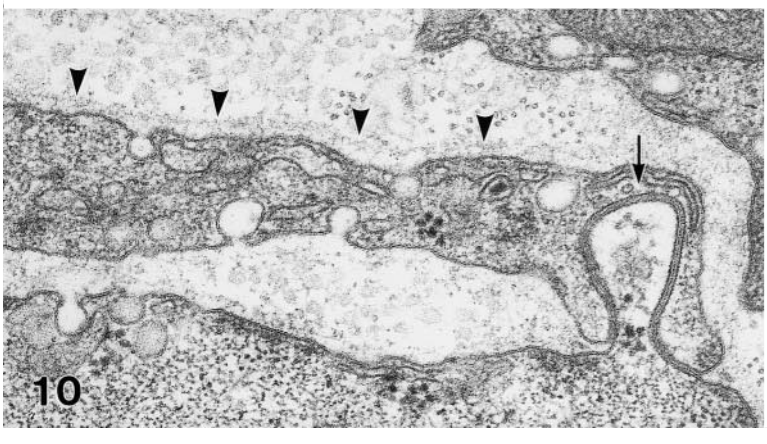
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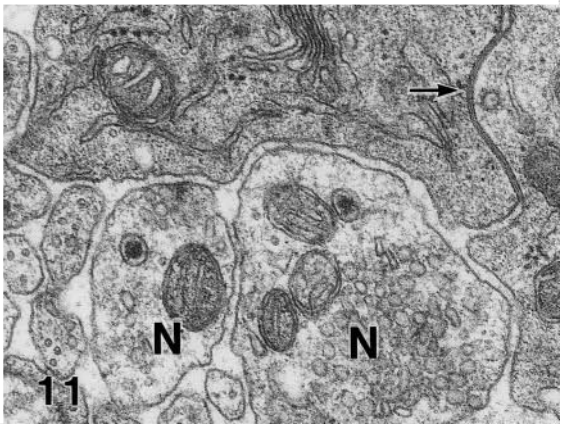
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somes are dispersed in the cytoplasm. Golgi apparatus can be seen. There is no basal lamina or caveolae. Microtubules, intermediate filaments, and thin filaments are indistinct. They often connect with smooth muscle cells by small gap junctions (Fig. 4, inset).

Cells of another type are characterized by numerous mitochondria and less electron-dense cytoplasm (Fig. 5). Golgi apparatus and smooth endoplasmic reticulum are well-developed. RER is also seen, but its cisterns rarely show a dilated form. Caveolae and basal lamina are not observed. They form gap junctions with each other (Fig. 5, inset) and with smooth muscle cells.

Ws/Ws rats. Interstitial cells and their processes are relatively sparse in the myenteric region of *Ws/Ws* rats (Fig. 6). Cells of the first type are observed without noticable change, like the +/+ rats (Fig. 7). It is worth noting that even a single fibroblast-like cell forms gap junctions with smooth muscle cells of both circular and longitudinal type in a single section (Fig. 7, inset). However, the second type of cells is greatly reduced in *Ws/Ws* rats. Examination of the number of interstitial cells on the montage micrographs reveals that 21 cells of the second type are counted along a length of 468 cross-sectioned smooth muscle cells bordering the myenteric plexus in +/+ rats, in contrast to only 2 cells observed along a length of 573 muscle cells in *Ws/Ws* rats. On the other hand, 16 and 23 cells of the first type are counted on the same montages in +/+ and *Ws/Ws* rats, respectively.

Interstitial cells in the DMP region

Deep muscular plexus (DMP) is located between two subdivisions of the circular muscle layer: an inner thin and an outer thick layer. Two types of interstitial cells are also recognized in this region. In contrast to those of the myenteric region, both types of interstitial cells in the DMP show no difference between +/+ and *Ws/Ws* rats. Thus, the following description applies only to the observation in the *Ws/Ws* rats.

Cells of the first type show features similar to those of the typical fibroblast. They are characterized by well-developed RER whose cisterns contain moderately dense material (Fig. 8). Golgi apparatus and free ribosomes are also observed. Cytoskeletal elements are inconspicuous in the cytoplasm. There is no basal lamina or caveola. They are often closely associated with nerve varicosities (Fig. 8) and form a few small gap junctions with the smooth muscle cells (Fig. 8, inset).

Cells of the second type are characterized by a number of mitochondria. The most conspicuous feature of this type of cell is the presence of large gap junctions connected with smooth muscle cells of the main layer and with one another (Figs. 9–11). Golgi apparatus, free ribosomes, and RER are also observed in the cytoplasm. However, cisterns of RER in this cell type rarely show a dilated form. Subsurface cisterns can be seen beneath the cell membrane. A continuous basal lamina and caveolae are clearly observed (Fig. 10). Microtubules, intermediate filaments, and thin filaments are also seen. These cells show close contacts with nerve varicosities (Fig. 11).

Discussion

The present study reveals that one type of gap junction-forming interstitial cell, characterized by low electron-dense cytoplasm, abundant mitochondria, no basal lamina and no caveolae in the myenteric region, is greatly reduced in the *Ws/Ws* rat small intestine, in contrast to fibroblast-like cells, which remain unchanged. Since the great reduction of their cell number is consistent with the loss of the immunoreactivity to anti-c-Kit antibody in the myenteric region of *Ws/Ws* rats, it can be concluded that those mitochondria-rich cells represent the c-*kit* expressing cells in the myenteric region, or ICC-AP in the rats. This type of cell seems to correspond to the mitochondria-rich cells previously described in the small intestine of the normal Wistar rats, which demonstrate a well-demarcated cell body and a few long slender cytoplasmic processes under the scanning electron microscope (Komuro 1989). This means that the present observation confirms that ICC-AP of the rat small intestine do not show any myoid features such as basal lamina or caveolae, and that they differ ultrastructurally from ICC-AP of the mouse intestine, which have been described as showing electron-dense cytoplasm, patch basal lamina and numerous caveolae (Thuneberg 1982; Huizinga et al. 1995; Ward et al. 1994,1995). Their ultrastructure also differs from those of ICC within the circular muscle layer of the rat stomach, which are characterized by the presence of many caveolae and electron-dense cytoplasm, as well as by large gap junctions and abundant mitochondria (Ishikawa et al. 1997). The marked increase in number of fibroblast-like cells reported in W/W^v mouse (Malysz et al. 1996) was not observed in *Ws/Ws* rats.

In contrast to the cells in the myenteric region, two types of interstitial cells in the DMP region were observed in *Ws/Ws* rats similar to those in their normal siblings, in spite of the absence of immunoreactivity to c-Kit. This fact makes it difficult to deduce which type of cells corresponds to c-*kit* expressing cells or ICC-DMP. However,

◀ **Fig. 8** A fibroblast-like cell located in the DMP region of *Ws/Ws* rat. Well-developed RER are conspicuous in the cytoplasm (*N* nerve bundles). ×17,000. *Inset* Higher magnification of the small gap junction between the cell of this type and smooth muscle cell. ×40000

Fig. 9 ICC-DMP of *Ws/Ws* rat, characterized by many mitochondria, caveolae (*arrowheads*), and gap junction (*arrow*) with a muscle cell. ×27000. *Inset* Higher magnification of the gap junction indicated by the *arrow*

Fig. 10 A cytoplasmic process of ICC-DMP forming a gap junction with a circular muscle cell (*arrow*). A distinct basal lamina is indicated by *arrowheads*. ×65000

Fig. 11 Axon terminals (*N*) containing synaptic vesicles closely associated with ICC-DMP of *Ws/Ws* rat. An *arrow* indicates a gap junction in the same type of cells. ×36000

fibroblast-like cells in both myeteric and DMP regions are characterized by exactly the same ultrastructural features, and are observed in *Ws/Ws* the same as in control animals. Thus, it can be speculated that another cell type characterized by a basal lamina, caveolae and many large gap junctions in this region corresponds to c-*kit* expressing cells there, or ICC-DMP in the rat small intestine.

The unchanged observation of ICC-DMP in *Ws/Ws* rats appears to indicate that they are able to develop and mature independently from the c-kit-SCF system, as suggested in the studies of W/W^v mouse (Malysz et al. 1996). Although it was reported that development of ICC-DMP showed different degrees of lesion, depending on timing and the number of postnatal injections of anti-c-Kit protein (ACK2) to BALB/c mouse (Torihashi et al. 1995), a normal distribution of ICC-DMP was also observed in Sl/Sl^d mouse (Ward et al. 1995).

Regarding the functional role of ICC, it is speculated that ICC-DMP are not involved in the generation of pacemaker activity, since they are observed in W/W^v mouse (Malysz et al. 1996) and Sl/Sl^d mouse (Ward et al. 1995), which lack normal activity of intestinal contraction and slow waves. The developmental study also revealed that electrical slow waves develop after the formation of an ICC-AP network, but before the appearance of ICC-DMP (Torihashi et al. 1997). The present observation of ICC-DMP in *Ws/Ws* rats, which have showed apparent abnormal activity in ileal contraction (Isozaki et al. 1995), seems to confirm this assumption, while ICC-AP are very likely to play an important role in the pacemaking function. Since ICC-DMP of the rat intestine have rich innervation and form many gap junctions with each other and with smooth muscle cells (Komuro and Seki 1995; Seki and Komuro 1997; the present study), they appear to function as a mediator of nerve signals to the smooth muscle, as suggested repeatedly in previous studies (Thuneberg 1982; Komuro et al. 1996; Sanders 1996). This assumption is compatible with the conclusion that ICC-DMP of the small intestine were most densely coupled with each other and with the circular muscle tissue via gap junctions, in comparison with the muscular tissue in the stomach and colon, by the immunohistochemical observation of c-*kit* expressing cells and gap junction protein (connexin 43) in the guinea pig gastrointestinal tract (Seki et al. 1998).

On the other hand, the present study demonstrates that both DMP and myenteric regions of the rat intestine contain fibroblast-like cells forming close contact with nerve varicosities and gap junctions with smooth muscle cells without apparent dependency on c-*kit*. Their cellular connection to both circular and longitudinal muscle cells by gap junctions is observed even in *Ws/Ws* rats, similar to those observed in normal Wistar rats (Komuro 1989). The gap junction-forming fibroblast-like cells were also observed within the circular muscle layer of the *Ws/Ws* rat stomach (Ishikawa et al. 1997). It is very likely that those fibroblast-like cells mediate electrical signals to muscle cells, though supporting data have not been available so far.

The cytological and functional heterogeneity of gap junction-forming interstitial cells, including ICC located in different tissue layers of different levels of digestive tract, is an important issue for future studies for a better understanding of the peculiar contractile activity of each organ.

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