Interstitial cells of Cajal in the gastrointestinal musculature of \textit{Wjic} c-kit mutant mice

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Abstract

Interstitial cells of Cajal (ICC) generate electrical rhythmicity and transduce neural signals in the gastrointestinal musculature. ICC express the proto-oncogene \textit{c-kit}, a receptor tyrosine kinase, and are identified morphologically by c-Kit immunoreactivity. The \textit{c-kit} gene is allelic with the murine white-spotting locus \textit{W}, and mutations of \textit{c-kit} are known as \textit{W} mutations. \textit{W} mutations affect various developmental aspects of hematopoietic cells, germ cells, melanocytes, mast cells and ICC. We examined \textit{Wjic/Wjic} mutant mice that have a mutation in the tyrosine kinase domain resulting in severe loss of protein function. \textit{Wjic/Wjic} homozygotes exhibited white coats and black eyes. The gross morphology of the gastrointestinal tract showed no abnormality in mutant mice other than a forestomach papilloma. In the stomach, intramuscular ICC (ICC-IM) were missing, and myenteric ICC (ICC-MY) were reduced in number. In the small intestine, the number of ICC-MY was severely reduced; however there was a normal distribution of deep muscular plexus ICC (ICC-DMP). In the cecum, the numbers of ICC-IM and ICC-MY were severely depleted. ICC-MY were almost entirely absent in the colon, whereas ICC-MY loss was restricted to the distal colon. Patterns of ICC deficiency were generally similar between \textit{Wjic/Wjic} mice and \textit{W/Wv} mutants, which lack a specific type of ICC. The enteric nervous system of the mutant mice appeared normal. From these findings, we conclude that \textit{Wjic/Wjic} mice represent a distinct, novel genotype resulting in a lack of a specific type of ICC in the gastrointestinal musculature.

Key words: \textit{W} mutant, c-Kit, interstitial cells of Cajal, gastrointestinal tract

Introduction

The proto-oncogene \textit{c-kit} encodes a receptor tyrosine kinase and is allelic with the murine white-spotting locus (\textit{W}) on chromosome 5 (Chabot \textit{et al.}, 1988; Geissler \textit{et al.}, 1988). c-Kit has an extracellular domain consisting 5 immunoglobulin-like repeats and a tyrosine kinase domain that is divided into 2 domains by an insert sequence. The expression of c-Kit is important in the development of hematopoietic cells, germ cells, melanocytes, mast cells, and interstitial cells of Cajal (ICC) (Maeda \textit{et al.}, 1992; Lennartsson \textit{et al.}, 2005). Many spontaneous mutations of the \textit{c-kit}
gene have been characterized in detail in W mutant mice. Mice heterozygous for mutant W alleles have depigmented areas of various sizes and patterns in their coats and homozygotes display several other phenotypes in addition to coat color. The W mutation results in the lack of the transmembrane region (amino acids 513-590) of c-Kit (Nocka et al., 1990). W/+ heterozygotes have a ventral spot and absence of pigments in the feet and tail tip. Most W/W homozygotes die in the perinatal stage with severe macrocytic anemia, and survivors are sterile and lack coat pigmentation (de Aberle, 1927). The W\(^n\) mutation results in an amino acid substitution from Thr to Met at position 660 (Nocka et al., 1990). W\(^n\)/+ heterozygotes have a diluted coat color in addition to a ventral spot. W\(^n\)/W\(^n\) homozygotes have large depigmented areas lacking any well-defined pattern. The W\(^v\) mutation results in an amino acid substitution from Thr to Met at position 660 (Nocka et al., 1990). W\(^v\)/+ heterozygotes have a diluted coat color in addition to a ventral spot. W\(^v\)/W\(^v\) homozygotes are viable, sterile, black-eyed-white mice with macrocytic anemia (Little and Cloudman, 1937). The W\(^v\)/W\(^v\) mutant allele was identified in the Central Institute for Experimental Animals (Kawasaki, Japan). W\(^v\)/W\(^v\) homozygotes have large depigmented areas lacking any well-defined pattern. The W\(^v\) mutation results in the substitution of Gly to Arg at position 595 of the tyrosine kinase domain of c-Kit causing severe reduction in kinase activity (Tsujimura et al., 1993). Indeed, cultured W\(^v\)/W\(^v\) mast cells demonstrate no detectable activity in an in vitro kinase assay.

ICC contribute to normal motility of the gastrointestinal (GI) tract, playing important roles in generating electrical rhythmicity (slow waves) and transducing neural signals (Sanders, 1996; Rumessen and Vanderwinden, 2003; Takaki, 2003; Hanani et al., 2005; Sanders and Ward, 2007). The morphology of ICC is unique and they form elaborate networks associated with enteric nerves in the GI musculature (Iino and Horiguchi, 2006). ICC can be divided into 3 groups based on morphological features and their position in the musculature (Sanders, 1996; Rumessen and Vanderwinden, 2003; Hanani et al., 2005). Intramuscular ICC (ICC-IM) have a bipolar form, lie along the intramuscular nerve fiber bundles, and are distributed within the circular and longitudinal muscle layers. Deep muscular plexus ICC (ICC-DMP) in the circular muscle are specialized ICC-IM localized to the small intestine and associated with nerve fibers in the deep muscular plexus (DMP). Myenteric ICC (ICC-MY) generate electrical slow waves and display a multipolar shape with several processes extending in various directions in the myenteric plexus layer. ICC on the submucosal surface of the circular layer (ICC-SM) have a multipolar shape and are distributed throughout the colon.

Because ICC depend on signaling through c-Kit for their development (Maeda et al., 1992), several different W mutant rodents, including W/W\(^n\) and W\(^n\)/W\(^n\) mice and W\(^s\)/W\(^s\) rats, have been investigated as potential models for the analysis of ICC function in vivo (Sanders and Ward, 2007). The utility of these animals as models for investigation of the function of ICC depends on the extent of their ICC deficiency. W/W\(^s\) mice are currently the most commonly used animal model for such investigations (Takaki, 2003). The reduced c-Kit function in these mice results in a lack of or reduction in ICC subtypes, e.g., ICC-MY throughout the GI tract and ICC-IM in the stomach and colon. We recently evaluated ICC distribution in W\(^v\)/W\(^v\) mice and observed an almost identical deficiency of ICC as that seen in W/W\(^n\) mice (Iino et al., 2007). In this study, we examined ICC distribution in W\(^v\)/W\(^v\) mutant mice that have a distinct c-Kit kinase dysfunction.
Materials and Methods

C57BL/6-W^nic/+ mice were provided by Dr. Y. Kitamura and M. Okabe (Osaka University) and maintained in our laboratory. +/+ and W^nic/W^nic mice were obtained by crossing W^nic/+ parents. All experiments used animals at 6 to 12 weeks after birth. The use and treatment of animals followed the Guidelines for Animal Experiments, University of Fukui Faculty of Medical Sciences. All efforts were made to minimize the number of animals used and their suffering. All mice were anesthetized with ether and the GI tract was dissected.

For cryostat studies, the GI tract was flushed with 0.01 M phosphate-buffered saline (PBS, pH 7.2), pinned to the Sylgard elastomer (Dow Corning Corporation, USA) floor of a dissecting dish, and then fixed with Zamboni’s fixative (2% paraformaldehyde and 1.5% saturated picric acid solution in 0.1 M phosphate buffer, pH 7.3) for 2 hours at room temperature. Following fixation, tissues were washed with PBS, immersed in 30% sucrose containing PBS, and embedded in OCT compound (Sakura Finetecnical Co., Japan). Cryostat sections were cut at 10 \( \mu \text{m} \) thickness using a Leica CM3050 cryostat and collected on poly-L-lysine-coated glass slides. For whole-mount preparations, tissues were placed in PBS, flushed with PBS, pinned to a dissecting dish, and stretched before being fixed in ice-cold acetone for 15 minutes. Tissues were washed with PBS and the mucosa removed by dissection. Specimens were then preincubated with normal donkey serum (5% in PBS) for 1 hour followed by overnight incubation with the following primary antibodies: rat anti-c-Kit (ACK4; Acris antibodies, Germany, 1:700), rabbit anti-protein gene product 9.5 (PGP9.5) (RA95101; UltraClone, England, 1:5,000). Specimens were then washed with PBS followed by incubation in secondary antibody (Alexa Fluor-coupled donkey anti-IgG; Molecular Probes, USA, 1:500) for 1 hour at room temperature, washing with PBS, counter staining with DAPI (Molecular Probes), and mounting with Mowiol solution.

Fluorescent images were examined using a Leica TCS-SP2 confocal microscope with excitation wavelengths of 350, 488 and 543 nm. Images were collected and composed using Leica Confocal Software. Some confocal micrographs were digital composites of several Z-series optical sections through the full or partial thickness of the musculature. Adobe Photoshop CS2 was used to compose the final plates.

For conventional electron microscopy, tissues from 3 animals were fixed using 3% glutaraldehyde and 4% paraformaldehyde in 0.1 M phosphate buffer (pH 7.2) for 2 hours at room temperature. Specimens were then postfixed with 1% OsO4, block-stained with uranyl acetate solution, and embedded in Epon 812. Ultrathin sections stained with uranyl acetate and lead citrate were examined under an electron microscope (Hitachi H-7650).

Results

Coat color (Fig. 1): From W^nic/+ parents, wild-type (+/+) and heterozygous (W^nic/+), and homozygous (W^nic/W^nic) mice were born at expected frequencies and grew to adulthoods. Wild-type mice had black coats, whereas heterozygotes had large depigmented areas lacking any well-defined pattern, and their pigmented areas appeared diluted. Homozygotes had completely white coats and mild growth retardation.
Gross-morphology of the GI tract: No gross morphological abnormalities were observed in the GI tract of \( \text{W}^{\text{ji}}/\text{W}^{\text{ji}} \) mice. The mucosal lumens of the GI tract also appeared normal, except for the stomach. In the forestomach of homozygotes, there were numerous papillomas demonstrating hyperplasia and hyperkeratosis of the epithelium (Fig. 2). However, these papillomas were not observed in forestomachs of \( \text{W}^{\text{ji}}/+ \) mice.

Immunohistochemical properties of GI musculature: We investigated sections and whole-
mount preparations to identify all types of ICC as reported in previous studies (Horiguchi and Komuro, 2000; Vanderwinden et al., 2000; Iino et al., 2007). Consistent with published results (Iino et al., 2007), in the +/+ mice, c-Kit-immunoreactive cells were observed throughout the GI tract, from the esophagus to the distal colon. In the W<sup>jic</sup>/W<sup>jic</sup> mice, the GI tract displayed a reduced number of c-Kit-immunopositive cells compared to +/+ mice (Fig. 3). Nerve fibers and nerve cell bodies in the enteric nervous system were examined and identified using the PGP9.5 antibody. There was no difference in PGP9.5 immunoreactivity between +/+ and W<sup>jic</sup>/W<sup>jic</sup> mice.

Esophagus: In +/+ mice, spindle-shaped ICC-IM with tapering processes were distributed

![Image of ICC distribution in the GI musculature.](image)
sparsely within the skeletal muscle region of the esophagus, and most were associated with nerve fibers. In \( W^{nic}/W^{nic} \) mice, there were no or few c-Kit-immunopositive cells in the esophageal musculature.

Stomach: In the stomach of \(+/+\) mice (Fig. 3A), ICC-IM were associated with nerve fiber bundles, as revealed by PGP9.5 immunoreactivity in the muscular layers. In whole-mount preparations, ICC-IM were spindle-shaped with long, thin processes extending the axes of smooth muscle cells. In addition to ICC-IM, a second type of ICC (ICC-MY) was present at the level of the myenteric plexus in the corpus and antrum of \(+/+\) mice (Fig. 3A). These cells possessed multiple thin extensions and interconnected to form a complex, dense network with other ICC-MY. The density of ICC-MY gradually increased from the proximal corpus to the antrum. In \( W^{nic}/W^{nic} \) mice (Fig. 3B), ICC-IM were not observed in the gastric musculature. Although ICC-MY were observed in whole-mount preparations of \( W^{nic}/W^{nic} \) mice (Fig. 4, A and B), the density was reduced compared to that in \(+/+\) mice. ICC-MY in the corpus (Fig. 4A) were sparsely distributed, and that in the terminal antrum (Fig. 4B) were numerous in \( W^{nic}/W^{nic} \) mice.

Small intestine: In the small intestine of \(+/+\) mice, cells with c-Kit immunoreactivity were observed in both the DMP and myenteric layers (Fig. 3C). ICC-DMP were bipolar in shape, had thin processes extending from cell bodies or main processes, and were orientated parallel to the long axis of circular muscle cells (Fig. 4D). ICC-MY had multiple cellular extensions forming a well-defined and highly branching network in the myenteric layer. The extensions of ICC-MY often had secondary branches that formed fine, highly interconnected regions of the ICC-MY network. In \( W^{nic}/W^{nic} \) mice, although ICC-DMP were observed at the same density as in wild-type mice (Fig. 4C), ICC-MY were not observed in the myenteric layer (Fig. 3D). In addition, ICC-DMP of \( W^{nic}/W^{nic} \) mice possessed a bipolar shape but lacked the short thin processes emanating from cell bodies or main processes (Fig. 4E).

Cecum: In the cecum of \(+/+\) mice, cells with c-Kit immunoreactivity were observed in muscular (ICC-IM) and myenteric layers (ICC-MY). ICC-IM had tapering bilateral extensions, and ICC-MY had multiple cellular extensions that formed a well-defined and highly branching network. In \( W^{nic}/W^{nic} \) mice, though ICC-IM were almost entirely absent, a small number of ICC-MY was observed with normal morphology.

Proximal colon: In the proximal colon of \(+/+\) mice, c-Kit-immunoreactive cells were observed within the circular and longitudinal muscle layers (ICC-IM), the myenteric layer (ICC-MY), and along the submucosal surface of the circular muscle layer (ICC-SM). ICC-IM possessed long processes that frequently bifurcated. ICC-MY had multiple extensions that branched to form a highly interconnected network, whereas ICC-SM were characterized by several thin processes interconnected by secondary processes to form a network. In \( W^{nic}/W^{nic} \) mice, ICC-IM were not observed in the muscle layers; ICC-MY were occasionally observed (Fig. 4F), and ICC-SM exhibited identical morphologies and distributions to those found in \(+/+\) mice.

Distal colon: In the distal colon of \(+/+\) mice, all types of ICC showed similar morphology and distribution patterns to those observed in the proximal colon (Fig. 3E). In \( W^{nic}/W^{nic} \) mice, although ICC-SM were identified at the submucosal surface of the circular layer (Figs. 3F and 4G), ICC-IM and ICC-MY were not observed (Fig. 3F).

Electron microscopic observations of \( W^{nic}/W^{nic} \) small intestine: We used electron microscopy
ICC in Wjic c-kit mutant mice

(Fig. 5) to observe typical ICC-DMP between the inner and outer layers of circular muscles in the ilea of Wjic/Wjic mice. These cells were characterized by numerous mitochondria, caveolae, a basal lamina, and large gap junctions. The ICC-DMP were connected to smooth muscle cells of the circular muscle layer, as well as to each other, by gap junctions. ICC-DMP were frequently associated with nerve fibers and nerve terminals. ICC-MY were not observed in the myenteric layer in the ilea of Wjic/Wjic mice.

Discussion

Several studies have investigated ICC distribution in c-kit mutant animals such as W/Wv and Wv/Wv mice (Horiguchi and Komuro, 2000; Vanderwinden et al., 2000; Takaki, 2003; Iino et al.,
S. Iino et al. (2007; Sanders and Ward, 2007). These studies have shown the loss of or decrease in specific types of ICC in the GI tract. In this study, we examined the GI tract of Wjic/Wjic mice that have not been previously investigated in terms of ICC. We found a similar pattern, to that observed in W/Wv or Wv/Wv mice (Iino et al., 2007), characterized by severe ICC loss (Table 1). For example, ICC-IM were absent in the stomach and colon; the numbers of ICC-MY in the GI tract were severely reduced, and the numbers of ICC-IM and ICC-MY in the cecum were also lower. In contrast, numbers of ICC-DMP in the small intestine and ICC-SM in the colon were unaffected in these mutant mice. Overall, among the 3 W mutants, Wjic/Wjic mice retained the lowest number of ICC in their GI musculature. Enteric nerves in the GI tract were observed to have normal distribution and morphology. From these studies, we conclude that the Wjic/Wjic mouse is a new model lacking a specific type of ICC.

The gross phenotypes of Wjic/Wjic, such as white coat, anemia, infertility, and papilloma in the forestomach are as expected from studies of other W mutants. Deficiency of melanocytes causes white coat color; reduction in the number of hematopoietic cells causes anemia, and deficiency of germ cells leads to infertility (Tsujimura et al., 1993). These symptoms are caused by the c-kit mutation and the decrease in c-Kit function during development of Wjic/Wjic mice. Forestomach papillomas are thought to be caused by the reflux of bile from the duodenum to the stomach in W/Wv mice (Yokoyama et al., 1982). The occurrence of forestomach papilloma in Wjic/Wjic mice also suggests gastroduodenal dysfunction, such as bile reflux, in these animals. Hence, Wjic/Wjic mice are a new model of not only ICC deficiency but also other phenotypes associated with c-Kit function deficiency.

Mutations at the W locus cause varying degrees of reduction in the kinase activity of c-Kit.
affecting aspects of hematopoiesis and proliferation and migration of primordial germ cells and melanoblasts (Reith et al., 1990); these mutations also influence the number of mast cells in W mutant mice. The absence of c-Kit kinase activity in W, W\textsuperscript{v}, W\textsuperscript{jic}, W\textsuperscript{42}, and W\textsuperscript{ps} mutants leads to disappearance of mast cells, and reduced kinase activity of c-Kit in W\textsuperscript{v} and W\textsuperscript{41} mutants causes a decrease in mast cell numbers (Nocka et al., 1990; Tsujimura et al., 1993). The kinase activity of c-Kit also affects the number of ICC in the GI musculature. Comparison of ICC distribution in W mutant mice with different kinase activities (W\textsuperscript{jic}/W\textsuperscript{jic} have no kinase activity; W/W\textsuperscript{v}, severely decreased activity; and W/W\textsuperscript{4}, moderately decreased activity) led us to conclude that the degree of ICC reduction depends on the degree of c-Kit kinase activity. Subpopulations of affected ICC are considered to require c-Kit activity for their development. ICC-IM in the stomach and colon and

|                | ICC-IM | ICC-DMP | ICC-MY | ICC-SM |
|----------------|--------|---------|--------|--------|
| Esophagus      |        | ++      |        |        |
| W/W\textsuperscript{v} |      |        |        |        |
| W/W\textsuperscript{v} |      |        |        |        |
| W\textsuperscript{42}/W\textsuperscript{jic} |      |        |        |        |
| Fundus         |        | ++      |        |        |
| W/W\textsuperscript{v} |      |        |        |        |
| W/W\textsuperscript{v} |      |        |        |        |
| W\textsuperscript{42}/W\textsuperscript{jic} |      |        |        |        |
| Corpus         |        | ++      | ++     |        |
| W/W\textsuperscript{v} |      |        |        |        |
| W/W\textsuperscript{v} |      |        |        |        |
| W\textsuperscript{42}/W\textsuperscript{jic} |      |        |        |        |
| Antrum         |        | ++      | ++     |        |
| W/W\textsuperscript{v} |      |        |        |        |
| W/W\textsuperscript{v} |      |        |        |        |
| W\textsuperscript{42}/W\textsuperscript{jic} |      |        |        |        |
| Small intestine |        | ++      | ++     |        |
| W/W\textsuperscript{v} |      |        |        |        |
| W/W\textsuperscript{v} |      |        |        |        |
| W\textsuperscript{42}/W\textsuperscript{jic} |      |        |        |        |
| Cecum          |        | ++      | ++     |        |
| W/W\textsuperscript{v} |      |        |        |        |
| W/W\textsuperscript{v} |      |        |        |        |
| W\textsuperscript{42}/W\textsuperscript{jic} |      |        |        |        |
| Proximal colon |        | ++      | ++     | ++     |
| W/W\textsuperscript{v} |      |        |        |        |
| W/W\textsuperscript{v} |      |        |        |        |
| W\textsuperscript{42}/W\textsuperscript{jic} |      |        |        |        |
| Distal colon   |        | ++      | ++     | ++     |
| W/W\textsuperscript{v} |      |        |        |        |
| W/W\textsuperscript{v} |      |        |        |        |
| W\textsuperscript{42}/W\textsuperscript{jic} |      |        |        |        |

++ shows existence of numerous ICC, + shows a small number of ICC and – shows no ICC in the musculature.
ICC-MY in the small intestine are subpopulations requiring high levels of c-Kit activity; other populations of ICC, such as ICC-DMP in the small intestine and ICC-SM in the colon, seem to be independent of c-Kit. Recently, we examined the expression of developmentally regulated genes using W/W mutant mice and found that ntrk2 and msx2 genes were downregulated (Horiguchi et al., 2010). These 2 genes were significantly suppressed through the developmental stages of the small intestine in W/W mice and are thought to regulate development of ICC-MY. To date, other genes regulating development of different ICC subtypes have not been elucidated. Further study is required to clarify the mechanisms of c-Kit independent ICC development using the various W mutants.

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