acta histochemica 112 (2010) 215–221

Contents lists available at ScienceDirect

acta histochemica

journal homepage: www.elsevier.de/acthis

Postnatal development of interstitial cells of Cajal in mouse colon in response to Kit signal blockade with Imatinib (Glivec®)

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ARTICLE INFO

Article history:
Received 7 January 2010
Received in revised form
7 February 2010
Accepted 8 February 2010

Keywords:
Colon
DOG1
Imatinib
SCF
ICC-SM
Mouse

ABSTRACT

This study investigated the response of interstitial cells of Cajal (ICC) in postnatal mouse colon to treatment with Imatinib (Glivec®, a potent inhibitor of Kit receptor). ICC were revealed by immunofluorescent staining on frozen cross-sections and whole-mount preparations by anti-Kit and DOG1 antibodies. Kit and p-Kit protein were also evaluated by Western blot. After administration of Imatinib for 4 days beginning at 8 days post-partum (P8), the mean density of Kit^+ ICC, which were localized around the myenteric nerve plexus (ICC-MY), within smooth muscle layers (ICC-IM) and in the connective tissue beneath the serosa (ICC-SS), was dramatically decreased to about 50% when compared with controls, but those Kit^+ cells located at the submucosal border of circular smooth muscle layer (ICC-SM) seemed to be unchanged in both cell number and morphology. A small number of $DOG1^+/Kit^-$ cells appeared during Imatinib administration. However, these Kit^+ ICC were not changed in mice even after 12 days of Imatinib treatment from P24. When Imatinib was discontinued, the number of ICC recovered to normal within 4 days. Our results indicate that the postnatal development of ICC in the mouse colon is Kit dependent, but ICC-SM are unlikely, and the Kit dependence of ICC development is also age-dependent.

Introduction

Interstitial cells of Cajal (ICC) are the pacemaker cells in the gastrointestinal tract and act as neurotransmission intermediates from the enteric nervous system to smooth muscle cells (Huizinga et al., 1995; Sanders, 1996; Ward and Sanders, 2001) and also serve as stretch sensors of the gastrointestinal (GI) tract (Sanders and Ward, 2007). ICC in the intestine can be divided into several subgroups according to their locations (Torihashi et al., 1994; Vanderwinden et al., 2000; Aranishi et al., 2009). They are found at: (1) the level of Auerbach's myenteric plexus (ICC-MY); (2) within the longitudinal and circular smooth muscle layers (ICC-IM); (3) around the deep muscular plexus of the small intestine (ICC-DMP); (4) at the border between the circular muscle layer and submucosa of the colon (ICC-SM); (5) in the connective tissue beneath the serosa of the colon (ICC-SS).

ICC express the gene product of *c-kit* (Ward et al., 1994; Huizinga et al., 1995), a proto-oncogene that encodes the receptor tyrosine kinase (Kit). Its ligand, stem cell factor (SCF), is produced by smooth muscle cells (SMCs) and neurons. It has been well documented that Kit signaling is essential for the proliferation and development of ICC in the small intestine during embryogenesis and during the neonatal period. Inactivation of Kit with neutralizing antibodies (Maeda et al., 1992; Torihashi et al., 1997, 1999) or Kit blocker (Beckett et al., 2007) in fetal and neonatal animals, or non-lethal mutations of Kit (Ward et al., 1994; Huizinga et al., 1995; Nakama et al., 1998) or SCF (Ward et al., 1995) in rats and mice, leads to malformation of ICC-MY, which may be the pacemakers in small intestine. However, the effects of Kit signaling on ICC in the colon during postnatal development are still not clear, despite the fact that considerable evidence has shown the absence and underdevelopment of ICC may closely associated with several colonic motility disorders in neonates, infants and adults. These include disorders such as: Hirschsprung's disease (Vanderwinden et al., 1996), pseudo-obstruction (Kenny et al., 1998), isolated hypoganglionosis (Rolle et al., 2002) and slow transit constipation (Wedel et al., 2002).

To investigate the Kit signaling effects on ICC, Imatinib (Glevic®), a potent inhibitor of Kit, which is used for the treatment of gastrointestinal stromal tumors (GIST) in clinical practice, was used in this experiment (Demetri, 2002). *In vitro* experiments have also shown that administration of Imatinib results in the inhibition of small intestinal motility in adult mice

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doi:10.1016/j.acthis.2010.02.003

(Shimojima et al., 2005) and humans (Popescu et al., 2006), and the disappearance of ICC in organ cultures of small intestinal tissue from the fetal and neonatal mice.

Therefore, we have used immunofluorescent staining and Western blot methods to investigate the alterations of ICC in the mouse colon at P8 (neonatal) and P24 (young) after Imatinib treatment.

Materials and methods

Animals

BALB/C mice were purchased from the Animal Center of the Third Military Medical University (Chongqing, China) and paired to produce offspring. Imatinib mesylate (Glivec®) was purchased from Novartis Pharma AG (Basel, Switzerland). Mice were intragastrically administered Imatinib at a dosage of 0.5 mg g^{-1} per day and animals were divided into 6 groups: (1) mice at P8 were killed 6 h after one dose of drug (n=3); (2) mice at P8 were treated with the drug for 1 day (n=5); (3) mice at P8 were treated with the drug for 4 days (n=8); (4) mice were administrated the drug for 4 days from P8 then it was withdrawn for 4 days (n=5); (5) mice at P24 were treated with the drug for 4 days (n=5); (6) mice at P24 were treated with the drug for 12 days (n=5). Glucose in water was given to 21 mice as controls. All experiments were performed in accordance with our University Health Guide for the Care and Use of Laboratory Animals.

Immunofluorescence

The entire colon from the ileo-cecal junction to the pelvic brim was carefully removed. For whole-mount preparations, the colon from groups 2–6 (n=25) and controls (n=15) was inflated with acetone for 30 min, then the mucosa was removed by sharp dissection using a dissection microscope. For frozen sections, the colon from group 3 (n=3) and control (n=3) was divided into three segments of identical length from oral to anal end: proximal, middle and distal colon, then each part was placed into optimal cutting temperature compound (OCT), and quickly frozen with liquid nitrogen. Longitudinal sections (6–8 μm thick) were cut with a cryostat (Leica CM 1850, Leica Microsystems, Wetzlar, Germany) and fixed with 100% acetone for 15 min (4 °C). The immunostaining procedures have been described previously. In short, whole mount preparations or frozen sections were incubated with primary antibody overnight at 4 °C and then secondary antibodies for 1 h at room temperature (Table 1). Sections were counterstained with DAPI. The stained results were detected by BX51 fluorescence microscope (Olympus, Tokyo, Japan) or TCS SP5 confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany) with an excitation wavelength appropriate for FITC(488 nm), Cy3(552 nm), Cy5 (625 nm) and DAPI(380 nm).

Table 1
The antibodies used in the study.

Antigen	Clone	Supplier	Dilution	Isotype	Conjugated
Kit	ACk2	eBioscience	1:100 IF	Rat IgG	Purified
Kit	Polyclone	Santa Cruz	1:1000 WB	Goat IgG	Purified
DOG1	Polyclone	Abcam	1:100 IF	Rabbit IgG	Purified
α-SMA	1A4	Boster	1:100 IF	Mouse IgG	Purified
p-Kit	Polyclone	Cell Signal	1:1000 WB	Rabbit IgG	Purified
β-actin	Polyclone	Santa Cruz	1:1000 WB	Mouse IgG	Purified
Anti-rat	Polyclone	Zymed	1:100 IF	Goat IgG	Cy3
Anti-rabbit	Polyclone	Zymed	1:100 IF	Goat IgG	Cy5
Anti-mouse	Polyclone	Dako	1:100 IF	Goat IgG	FITC
Anti-goat	Polyclone	Dako	1:2000 WB	Donkey IgG	HRP
Anti-rabbit	Polyclone	Dako	1:3000 WB	Goat IgG	HRP
Anti-mouse	Polyclone	Dako	1:1000 WB	Goat IgG	HRP

Western blotting

Total protein was extracted from smooth muscle layers of the colon from group 1 (n=3) and control (n=3) using RIPA lysis buffer (50 mM tris pH 7.4, 150 mM NaCl, 1% triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mM EDTA, 1 mM sodium orthovanadate, 10 mM NaF), containing 10 μg/ml aprotinin, 10 μg/ml leupeptin and 1 mM phenylmethylsulphonyl fluoride. The protein concentration was measured using a BCA Protein Assay Kit (Beyotime Biotechnology, Jiangsu, China). Total protein (100 μg) was separated on 7.5% SDS-PAGE and transferred to a PVDF membrane at 350 mA for 1.5 h by using semi-dry transfer cell (Bio-Rad, Hercules, CA, USA). Then the membrane was incubated in blocking buffer consisting of 5% non-fat dry milk for 2 h at room temperature, and then with primary antibodies overnight at 4 °C and secondary antibodies subsequently (Table 1). Protein–antibody complexes were detected with an ECL Western blotting detection and analysis system (GE Healthcare Life Sciences, Amersham Biosciences, Bucks., UK).

Measurements and statistical analysis

Six intestinal segments were sampled in a random manner from experimental animals for whole mount preparations. After immunofluorescent staining assessment, photomicrographs of both types of Kit positive cells were taken in 10 random fields ($\times$200 magnification, 0.2607 mm^2) per whole-mount preparation with a digital camera (SPOT, Diagnostic Instruments, Sterling Heights, MI, USA) mounted on a BX51 fluorescence microscope (Olympus, Tokyo, Japan). The numbers of Kit^+ cells were counted with Image-Pro Plus 5.0 (Media Cybernetics, Silver Spring, MD, USA). Data were expressed as means $\pm$ S.E.M. The n value reported in the text refers to the number of animal used. Differences in the data were evaluated by student t test, and $P < 0.05$ was taken as a statistically significant difference.

Results

Kit signaling blockade by Imatinib administration

To evaluate the Kit blocking effects of Imatinib, Western blot was applied to assay the alterations of phosphorylated Kit protein (p-Kit) and Kit. The results showed that the amount of p-Kit was obviously decreased and Kit expression was persistent after Imatinib treatment for 6 h (Fig. 1), that means Imatinib can inhibit Kit signaling by inhibition of Kit phosphorylation and this drug can be used *in vivo* to investigate the effects of Kit blockade on ICC.

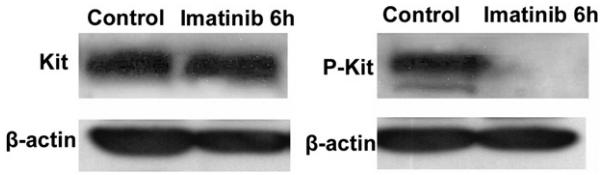

Fig. 1. Western blotting showed that Kit and p-Kit can be detected in control. Six hours after one dose of Imatinib, p-Kit was nearly not detected as compared with Kit expression.

Distribution alterations of ICC in neonatal mouse colon

To show clearly the alterations of ICC distribution after Kit blockade, Kit/α-smooth muscle actin (α-SMA) double labeling was carried out on sections of proximal colon. The results showed Kit^+ ICC were seen apparently between the submucosa and circular muscle layer (ICC-SM), at the level of Auerbach's plexus (ICC-MY), within the smooth muscle layers (ICC-IM) and in the connective tissue beneath the serosa (ICC-SS) in the controls (Figs. 2A–C). However, after administration of the drug for 4 days beginning at P8, the Kit^+ ICC-MY, ICC-IM and ICC-SS were dramatically decreased, apart from Kit^+ ICC-SM that were still present (Fig. 2D–F).

Morphological changes of ICC in neonatal mouse colon

To clarify further the alterations in morphology and cell number, Kit immunofluorescent staining on whole mount preparations of proximal colon was performed. Numerous Kit^+ ICC-MY, ICC-SS and ICC-SM with round cell bodies and long processes were seen, and ICC-IM in parallel with smooth muscle cells (Figs. 3A–D). After administration for 1 day, Kit^+ ICC-IM, ICC-MY and ICC-SS were characterized by shortened processes and disrupted cellular network (Fig. 3E–G). After treatment for 4 days with Imatinib, the cellular network of ICC-MY was more obviously disrupted and ICC-IM and ICC-SS were greatly decreased in cell number (Fig. 3I–K). After withdrawal of the drug, ICC-MY, ICC-IM and ICC-SS mainly recovered to normal within 4 days (Figs. 3M–O). However, it is noteworthy that Kit^+ ICC-SM seemed neither to change in morphology nor in cell number during drug administration and withdrawal (Fig. 3D, H, L and P).

Kit expression may be down-regulated in the condition of Kit signaling blockade, and DOG1, a Ca(2+)-activated Cl(−) channel, which is also named Ano1 or TMEM16A, was also the marker of ICC. So we investigated the expression of DOG1 as cellular markers of ICC. Immunofluorescent staining showed that the $DOG1^+$ were also Kit^+ in controls (Figs. 4A–C), and a small number of $Kit^-/DOG1^+$ and $Kit^{weak}/DOG1^+$ cells were observed after 1 day administration (Fig. 4D–E). After administration for 4 days, a dramatic reduction of Kit^+ ICC were observed, which were also DOG1 positive (Figs. 4G–I). One explanation of this result might be that down-regulation of Kit expression is involved in the process of ICC lost after Kit blockade.

Density alteration of ICC in neonatal mouse colon

To understand better the degree of Kit^+ ICC reduction, we counted the cell numbers of ICC subgroups individually in the proximal colon on whole-mount preparations. The results indicated that Kit^+ ICC-IM in the proximal colon decreased from $7.90 \pm 0.21 \times 10^2$ to $2.21 \pm 0.09 \times 10^2$ mm^{-2} (Table 2) at 4 days after drug administration from P8, and similarly the mean density of Kit^+ ICC-MY decreased from $17.7 \pm 0.21 \times 10^2$ mm^{-2} to a value less than a half ($7.51 \pm 0.09 \times 10^2$ mm^{-2}) (Table 2). The cell numbers of both Kit^+ ICC-IM and ICC-MY nearly recovered to normal within 4 days. However, those Kit^+ ICC-SM were not changed in density during drug administration and withdrawal (Table 2).

Effects of Kit blockade on ICC in young mouse colon

Kit immunofluorescent staining showed that the Kit^+ ICC-MY, ICC-IM, ICC-SM and ICC-SS showed no change in either cell numbers or morphology after either treatment for 4 days (Figs. 5A–D) or 12 days with Imatinib as compared with controls (Fig. 5E–H) from P24. It can be interpreted that ICC in young mice may be much less sensitive to Kit blockade than neonatal ones.

Discussion

The present study demonstrates that: (1) postnatal development of ICC in the mouse colon is dependent on Kit signaling, but this may not be the case with ICC-SM; (2) ICC become insensitive to Kit blockade in older mice; (3) Imatinib can be used as a potent Kit signal blocker *in vivo*.

Kit signal blockade effects by Imatinib

Imatinib was developed as a potent inhibitor of Bcr–Abl tyrosine kinase in chronic myeloid leukemia (CML), platelet-derived growth factor receptor kinase (PDGFR) and also Kit tyrosine kinase (Manley et al., 2002). ICC express neither Bcr–Abl tyrosine kinase, nor PDGFR (Iino et al., 2009). To further confirm the Kit blocking effect by intragastric administration of Imatinib, Western blotting was carried out to assay the level of Kit protein phosphorylation (p-Kit). The results showed that p-Kit was

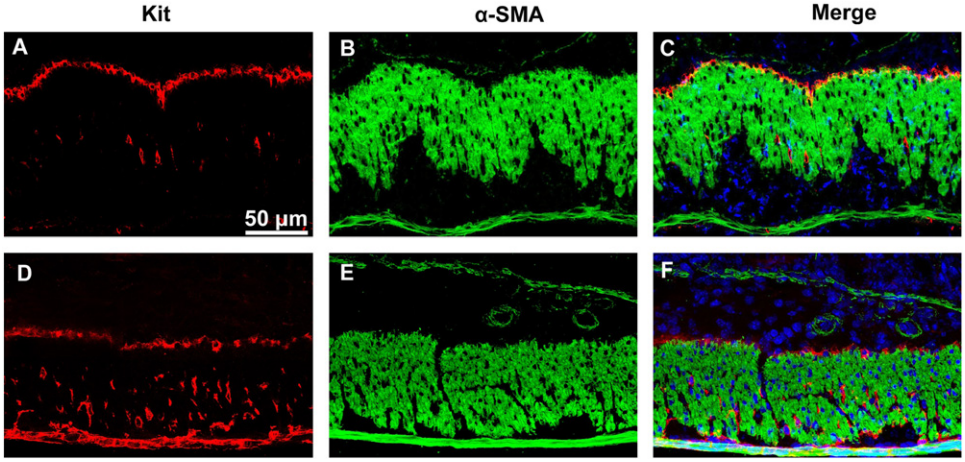

Fig. 2. Confocal images of ICC in the proximal colon labeled with Kit after Imatinib treatment for 4 days from P8 on longitudinal sections. (A–C) Kit positive cells were located between (ICC-MY) and within the longitudinal and circular smooth muscle layers (ICC-IM), at the border between circular muscle layer and submucosa of colon (ICC-SM), and in the connective tissue beneath serosa of colon (ICC-SS) in control. (D–F) After treatment for 4 days, ICC-MY, ICC-IM and ICC-SS were greatly decreased, while ICC-SM were normal present. Scale bar A–F=40 μm.

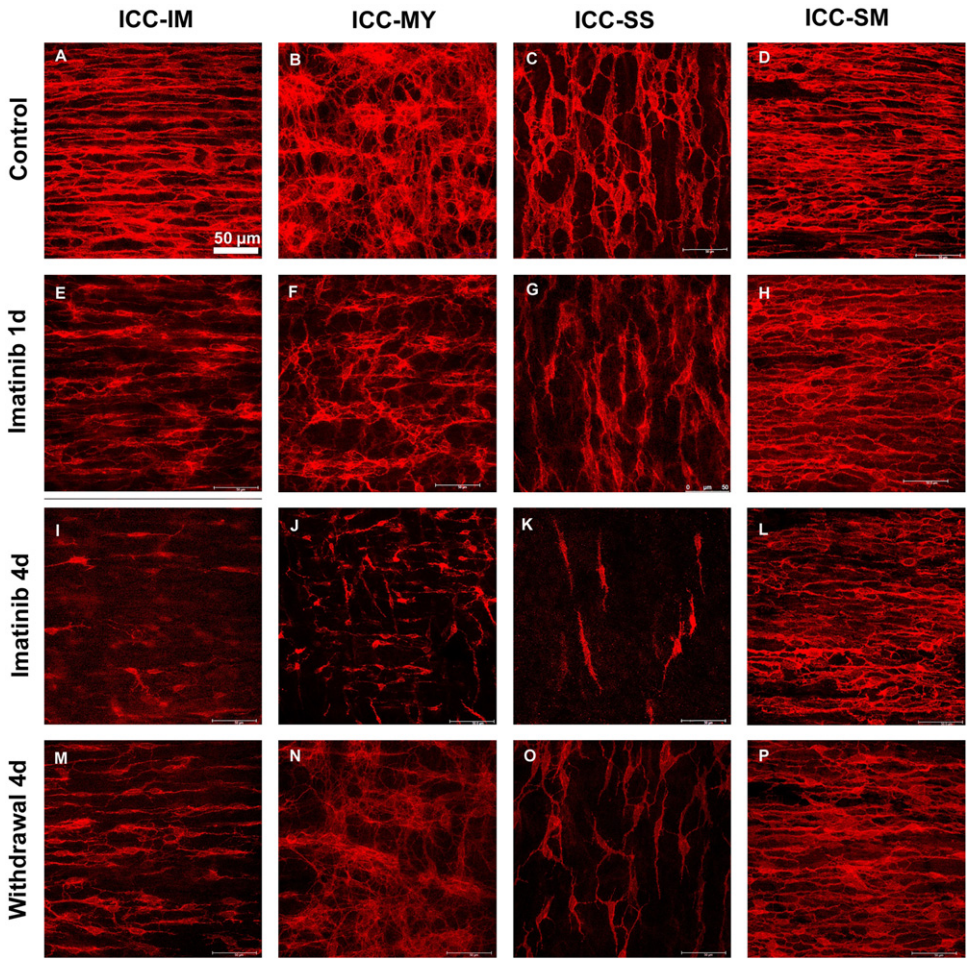

Fig. 3. Confocal images of ICC in proximal colon labeled with Kit on whole-mount preparations showing the alterations of distribution and morphology in control (A–D), after administration for one (E–H) and 4 days (I–L), and withdrawal for 4 days (M–P). ICC-IM, ICC-MY, ICC-SS and ICC-SM were normally present with famose processes and intact cellular networks (A–D). (E–H) One day after administration, the processes of ICC-IM, ICC-MY and ICC-SS were obviously shortened and decreased, except that ICC-SM were not changed. (I–L) The density of ICC were obviously reduced in cell numbers at the level of ICC-IM, ICC-MY and ICC-SS, and ICC-SM seemed to be still normally present. (M–P) All the subgroups of ICC almost recovered to normal after withdrawal for 4 days in cell number and morphology. Scale bar A–P=50 μm.

predominantly inhibited suggesting a potent inhibitive effect of Imatinib on Kit signaling *in vivo*, so that the effects of Imatinib on ICC in mouse colon may be induced by a Kit blocking action.

Kit dependent development of ICC in neonatal colon

ICC progenitors begin to express Kit in the foregut on embryonic day 12 (E12) and in the hindgut of mouse on E14 and then the Kit expression persists throughout the entire life (Torihashi et al., 1995; Faussone-Pellegrini et al., 1996; Ward et al., 1997; Wu et al., 2000). It has been considered that Kit signaling is essential for the genesis, development and proliferation of ICC in the small intestine by using anti-Kit monoclonal antibody or mice with non-lethal mutation of the *c-kit* gene (W/W^{v}) or SCF gene (Sl/Sl^{d}) (Ward et al., 1994, 1995; Torihashi et al., 1995; Huizinga et al., 1995; Kluppel et al., 1998; Beckett et al., 2007). However, Bernex et al. (1996) prefer to consider that Kit signaling is only required for the postnatal development of ICC in the small intestine. Our results showed that after Kit signaling blockade, Kit^{+} ICC located at the level of ICC-MY, ICC-IM and ICC-SS decreased dramatically after administration of Imatinib for 4 days starting at P8 on both sections and on whole-mount preparations. This indicates the postnatal development of ICC in mouse colon is Kit dependent as well as those in the small intestine. Moreover, our results showed that ICC in the colon of young mice were insensitive to Kit signal blockade as compared with that of neonatal ones, indicating the Kit dependence of ICC development is also age-dependent, like those in the small intestine, which are also Kit- and age-dependent. A long-term Kit signal blockade (>15 days) is needed to induce ICC loss in the small intestine of adult animals (Beckett et al., 2007; Mei et al., 2009b).

Distinct responses of ICC-SM to Imatinib treatment

ICC can be divided into four subgroups according to their location in the colon: ICC-MY, ICC-IM, ICC-SM and ICC-DMP, and they play different roles in the regulation of colonic motility. ICC-MY and ICC-IM in the colon of W/Wv mice were severely disrupted, but only ICC-SM remained unchanged (Okishio et al., 2005). This means that the genesis of ICC-SM in the colon is independent of Kit signaling. ICC-SM in the colon are still present after anti-Kit antibody treatment as compared with ICC-MY and ICC-IM in the colon (Torihashi et al., 1995), but the alterations in morphology and quantities of ICC-SM after Kit signaling blockade are still unclear in the colon. Our results showed that ICC-IM, ICC-MY and ICC-SS decreased obviously in cell numbers and disrupted in cellular networks after Kit blockade for 4 days from P8, but ICC-SM remained unchanged in morphology and cell number. This indicates that the postnatal development of ICC-SM may be

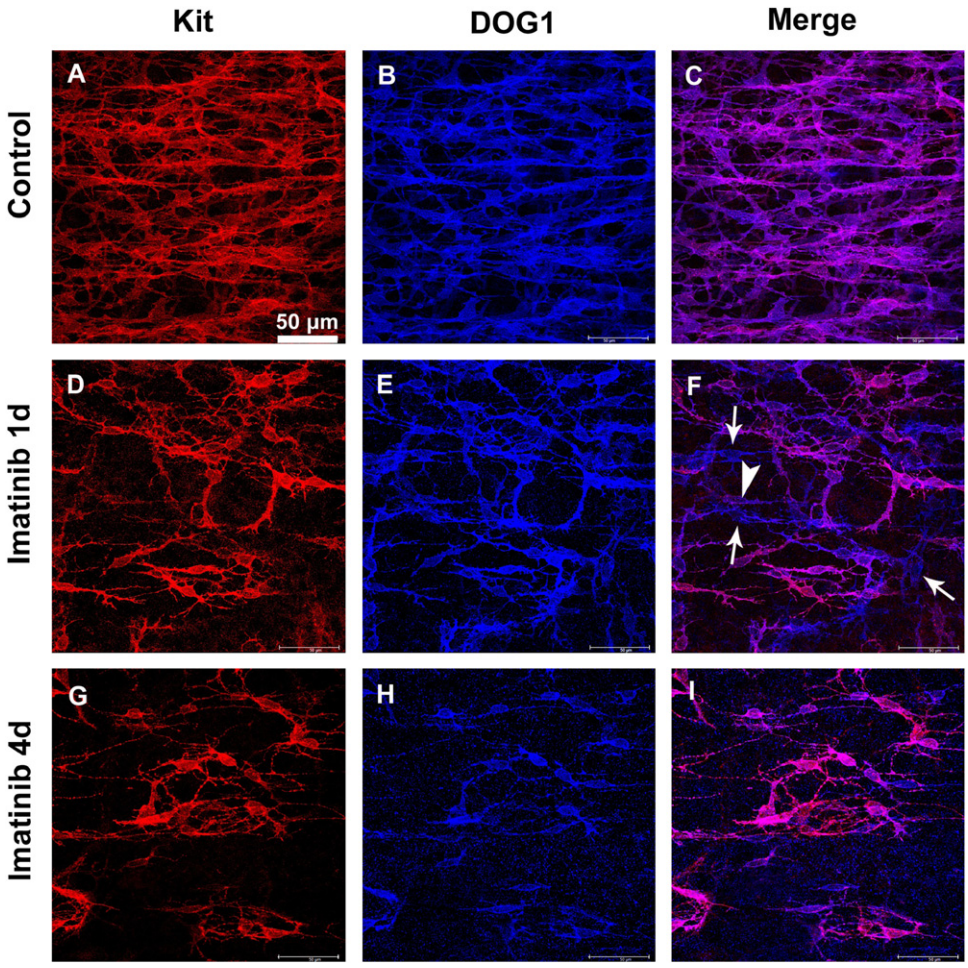

Fig. 4. Confocal images of ICC-MY in proximal colon labeled with Kit and DOG1 on whole-mount preparations in control (A–C), after administration for 1 days (D–F) and 4 days (G–I) from P8. (A–C) Kit and DOG1 were co-localized in ICC well in control. (D–F) After 1 day of Imatinib treatment, a small number of Kit^{weak}/DOG^{+} (arrowhead) and $Kit^{-}/DOG1^{+}$ (arrows) cells were observed. (G–F) Such cells were not present, although there was a dramatic decrease of cell number after 4 days' treatment. Scale bar A–I=50 μm.

Table 2
The mean density of ICC-IM, ICC-MY and ICC-SM in the proximal colon during Imatinib administration and withdrawal from P8.

	24 h ($\times 10^2$ mm^{-2})		**96 h** ($\times 10^2$ mm^{-2})		**48 h** ($\times 10^2$ mm^{-2})	
	Control	**Imatinib**	**Control**	**Imatinib**	**Control**	**Withdrawal**
ICC-SM	3.53 ± 0.12	3.62 ± 0.12	3.45 ± 0.10	3.42 ± 0.11	3.37 ± 0.12	3.40 ± 0.12
ICC-IM	7.90 ± 0.21	7.54 ± 0.22	7.86 ± 0.20	2.21 ± 0.09[ab]	7.96 ± 0.18	5.16 ± 0.12[ab]
ICC-MY	17.7 ± 0.21	15.6 ± 0.22[a]	15.4 ± 0.20[b]	7.51 ± 0.09[ab]	14.6 ± 0.18	13.5 ± 0.12[b]

Data given as mean ± S.E.M.
N=3 representing numbers of mice used.

[a] Significantly different from control at the same time-point ($P < 0.05$).
[b] Significantly different from the earlier time-point (i.e., 96 h vs 24 h) ($P < 0.05$).

independent of Kit signaling. Our previous results have shown that postnatal development of ICC-SM appear much later than other ICC subgroups in the colon and increase in total cell numbers, but no proliferative markers are detectable that are totally different from the other ICC subgroups (Han et al., 2010). Similarly, ICC-MY were obviously disrupted, but ICC-DMP showed normal organization and numbers in the small intestine of W/Wv mice (Malysz et al., 1996). Therefore, it seems that distinct ICC subgroups in the GI tract vary in biological characteristics and developmental processes, which suggests that different subgroups of ICC should be studied individually.

Loss and reappearance of Kit positive cells

The disappearance of ICC following Kit signal inhibition raises an important question as to whether there is a real reduction of ICC in neonatal mouse colon during Imatinib treatment. ICC express both CD44 and DOG1, as well as Kit, which can be taken as the markers besides Kit protein (Gomez-Pinilla et al., 2009; Yu et al., 2009). It has been suggested that ICC do not undergo apoptosis, but transdifferentiate toward a smooth muscle phenotype in the small intestine after Kit signal blockade (Torihashi et al., 1999; Mei et al., 2009b). Therefore, we used immunofluorescent staining with DOG1

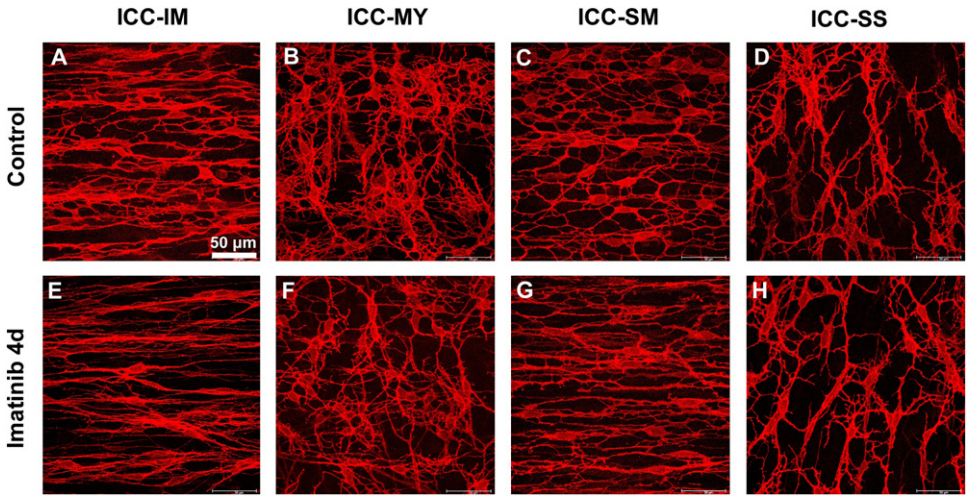

Fig. 5. Confocal images of ICC in the proximal colon of mice in control (A–D) and Imatinib treatment for 4 days (E–H) from P24 on whole mount preparations, and neither ICC subgroup changed after administration of Imatinib for 4 days. Scale bar, A–H=50 μm.

antibody, and the results showed a small number of $Kit^{-}/DOG1^{+}$ and $Kit^{weak}/DOG1^{+}$ appeared after day 1 of Imatinib administration and then these cells could not been observed after treatment for 4 days. Moreover, immunofluorescent staining with anti-CD44 antibody also showed a similar alteration (data not shown). These results may suggest that the reduced Kit^{+} ICC were not a real reduction of ICC, but they may cease expressing DOG1 and CD44 as well as Kit and transdifferentiate to a kind of intermediate cells. So new cellular markers, which ICC persistently express during Kit signal blockade, and electronic microscope, gold standard for the identification of ICC, are necessary to investigate the fate of lost ICC.

Our previous results have shown that proliferation is involved in the recovery of ICC after loss in adult animals (Mei et al., 2006, 2009a, 2009b) and ICC in mouse colon and small intestine proliferate during postnatal development (Mei et al., 2009c; Han et al., 2010). The present study showed that ICC recovered to normal after withdrawal of Imatinib and whether proliferation is involved in this recovery process requires further investigation.

Our study provides evidence that the postnatal development of ICC in the colon is Kit dependent, suggesting that insufficiency of Kit signaling would lead to the underdevelopment of ICC, which might be one of the mechanisms underlying the motility disorders of neonates and infants.

Acknowledgements

We thank Wei Sun and Li-ting Wang (Central laboratory, Third Military Medical University) for their help with the confocal laser scanning microscopy. This study was supported in part by the Natural Science Foundation Project of CQ CSTC 2009BB5320.

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