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ORIGINAL ARTICLE

Apoptosis of interstitial cells of Cajal, smooth muscle cells, and enteric neurons induced by intestinal ischemia and reperfusion injury in adult guinea pigs

Feng Mei · Sheng Guo · Yang-tao He · Jiang Zhu · De-shan Zhou · Jian-qin Niu · Han-zhi Wang · Yan-ping Tian

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Abstract This study aimed at evaluating whether apoptosis of interstitial cells of Cajal (ICC), smooth muscle cells (SMC), and enteric neurons was involved in a guinea pig model of intestinal ischemia and reperfusion injury. The small intestinal segments were resected at either 6 (I_{60}/R_{6h}) and 12 h (I_{60}/R_{12h}) or 7 (I_{60}/R_{7d}) to 14 (I_{60}/R_{14d}) days after 60 min intestinal ischemia in the adult guinea pigs and studied by immunohistochemistry with anti-Kit, 5-bromo-2′-deoxyuridine (BrdU), α-smooth muscle actin, vimentin, and β-tublin III antibodies. Also, apoptosis was tested by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method. In the I_{60}/R_{12h} injury, there was a ~50% decrease of Kit+ cells in cell numbers at the level of myenteric plexus and a number of Kit-/vimentin-positive cells were labeled by TUNEL. Also, a few SMC and enteric neurons were TUNEL positive. The Kit+ ICC recovered to normal and a number of Kit-/BrdU-double-positive cells were observed in the I_{60}/R_{14d} group. Our results indicated that the intestinal I/R injury could lead to apoptosis of ICC, SMC, and enteric neurons which may contribute to the gastrointestinal motility disorders, and proliferation was involved in the recovery of ICC.

Keywords Proliferation · ICC-MY · ICC-DMP · TUNEL · BrdU

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Feng Mei and Sheng Guo contributed equally to this work.

F. Mei (✉) · Y.-t. He · D.-s. Zhou · J.-q. Niu · H.-z. Wang · Y.-p. Tian
Department of Histology and Embryology,
Third Military Medical University,
Chongqing 400038, China
e-mail: thethirdmilitary@sohu.com

S. Guo
Department of Immunology, Third Military Medical University,
Chongqing 400038, China

J. Zhu
Department of Pathology, Southwest Hospital,
Third Military Medical University,
Chongqing 400038, China

D.-s. Zhou
Department of Histology and Embryology,
Capital University of Medical Sciences,
Beijing 100054, China

Introduction

The small intestine is one of the most susceptible organs to ischemia and reperfusion (I/R) injury such as is often encountered during hemorrhagic shock or small bowel transplantation. In an intestinal I/R injury model, changes in mucous and systemic inflammatory responses have been documented [1]. Some studies have also shown changes in gastrointestinal motility [2–6]; however, the underlying mechanisms are not well understood yet.

Interstitial cells of Cajal (ICC) play an important role in the regulation of gastrointestinal (GI) motility [7–9]. ICC in the small intestine are divided into two subgroups. One subgroup is located at the level of the myenteric plexus (ICC-MY) and acts as the pacemaker cells that generate and propagate the slow waves in the small intestine [10, 11]. The other is closely associated with the deep muscular plexus (ICC-DMP), and these cells play a role in the mediation of inputs from the enteric nervous system to GI smooth muscles [12–14].

ICC express the product of the *c-kit* gene [11, 15, 16], a proto-oncogene that encodes the receptor tyrosine kinase (Kit) which is considered essential for the development, differentiation, and functional maintenance of ICC [17]. A

recent study has also shown that a downregulated ICC Kit expression is probably associated with the gastrointestinal motility disorders resulting from I/R injury of the small intestine [2]. Other studies have also shown the loss of Kit immunopositivity after intestinal surgical manipulation [18] and gastrointestinal disorders, such as intestinal obstruction [19, 20], and this loss is probably one of the underlying mechanisms associated with intestinal motility dysfunction. However, it is noteworthy that apoptosis is generally observed in the liver [21], kidney [22], intestine [23], and lung [24] after I/R injuries, but it is not clear whether apoptosis of the ICC, smooth muscle cells (SMC), or enteric neurons is also involved in the intestinal I/R injury. Furthermore, a reexpression of Kit protein may contribute to the recovery of ICC networks after disrupted by intestinal disorders [18–20]. Also, a proliferative event has been reported during the recovery of the ICC after disruption by intestinal transection and anastomosis [25], but it is unclear whether the proliferation is linked to the recovery of the ICC after an I/R injury as well as the Kit reexpression. This study investigated alterations in the morphology and number of ICC in an intestinal I/R injury model using immunofluorescence, detection of apoptosis by terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) methodology, and detection of cell proliferation using 5-bromo-2′-deoxyuridine (BrdU) incorporation.

Materials and methods

Animals

Twenty-four adult guinea pigs (300–350 g, male or female, aged 6–8 weeks) were purchased from the Animal Center of the Third Military Medical University (Chongqing, China) and used in our experiments. All experiments were performed in accordance with our University Health Guide for the Care and Use of Laboratory Animals.

Animal surgery

Operations for experimental animals were performed under pentobarbital sodium anesthesia (Nembutal 50 μg g^{-1}). After the laparotomy by midline incision, the vascular arcades were ligated between the descending branch of the right colic artery and the ascending branch of the ileocolic artery, between the descending branch of the ileocolic artery and the last ileal artery, and between the jejunal arteries just proximal and distal to the point of the superior mesenteric artery in order occlude and interrupt the collateral blood flow. In addition, bulldog clamps were applied to the jejunum and terminal ileum at the same ligation levels as the vascular arcades in order to interrupt collateral flow within the intestinal wall. Then, the superior mesenteric artery was occluded just proximal to the right colic artery by vascular microclip. The right colic artery and vein and the ileocolic artery and vein were also occluded (supplemental Fig. 1). After the planned ischemic period, the vascular microclips and bulldog clamps were removed and blood reperfusion of the intestine confirmed by an immediate change of intestinal color from white to pink and the recovery of mesenteric arterial pulsations. The intestines were carefully returned to the abdomen and the incision was closed by two layers of continuous 3–0 silk sutures.

Experimental groups

Animals were divided into four groups according to the ischemia and reperfusion periods: (1) ischemia for 60 min followed by reperfusion for 6 h (I_{60}/R_{6h}), (2) ischemia for 60 min followed by reperfusion for 12 h (I_{60}/R_{12h}), (3) ischemia for 60 min followed by reperfusion for 7 days (I_{60}/R_{7d}), and (4) ischemia for 60 min followed by reperfusion for 14 days (I_{60}/R_{14d}; N=3 in each group). Sham control animals (N=3 in each group) were treated in an identical fashion except for vascular clamping. At the end of each experiment, the animals were killed by an overdose of pentobarbital and the ileum was harvested.

Immunofluorescence

The ileum was removed and enteric contents were washed away with phosphate-buffered saline (PBS). The small intestine was inflated with acetone for 30 min (room temperature), then opened along the mesenteric border, then the mucosa was removed, and the longitudinal smooth muscle layer containing the ICC-MY (also referred to as the ICC associated with Auerbach's plexus or ICC-MP by some authors) and the circular smooth muscle layer associated with ICC around the deep muscular plexus were prepared with the aid of a dissection microscope. The immunostaining procedures have been described previously [26]. Briefly, ICC were identified by using a rat monoclonal antibody raised against Kit (ACK2, 5 μg ml^{-1}; eBioscience) and immunoreactivity was detected by using a Cy3-conjugated secondary antibody (antirat IgG, 1:100; Zymed). Vimentin or smooth muscle actin was detected with a mouse monoclonal vimentin (1:100; DAKO) or α-smooth muscle actin (α-SMA; 1:100; Santa Cruz) antibody, and enteric neurons were detected with a rabbit polyclonal β-tublin III antibody (1:100; Sigma) respectively using the same procedures. Antibody-positive cells were labeled by a Cy5-conjugated secondary antibody (antimouse IgG, 1:100; Zymed), a fluorescein isothiocyanate (FITC)-conjugated (antimouse IgG, 1:100; DAKO), or a tetramethylrhodamine isothiocyanate (TRITC)-conjugated secondary antibody (antirabbit IgG, 1:100; Zymed). Nega-

tive control specimens were prepared in the same manner, but each primary antibody was omitted. The stained results were examined using a BX51 fluorescence microscope (OLYMPUS, Japan) or with a TCS SP5 confocal laser scanning microscope (Leica, Germany) with an excitation wavelength appropriate for FITC (488 nm), Cy5 (650 nm), Cy3 (552 nm), or TRITC (552 nm). The Z stacking of confocal images at 3- to 5-μm intervals contained all the levels of positively stained cells and processes.

Detection of ICC apoptosis

To detect the apoptosis of ICC, TUNEL labeling was done using an apoptosis detection Kit (Roche). Whole-mount preparations were fixed with 4% paraformaldehyde for 20 min at 25°C, washed twice with PBS for 30 min, and then treated with 0.1% Triton X-100 for 20 min on ice (2–8°C). The whole-mount preparations were incubated with TUNEL reaction buffer containing 45 μL of the labeling solution and 5 μL of the enzyme solution at 37°C for 1 h in a humidified atmosphere in the dark. Sections were then washed three times for 15 min each with PBS to remove unincorporated fluorescein-dUTP. The specimens were observed with a fluorescence microscope with an excitation wavelength in the range of 450–500 nm.

Detection of proliferating ICC

To identify the proliferation of ICC, daily intraperitoneal injection of BrdU (10 mg kg^{-1} day^{-1}, Sigma) was given

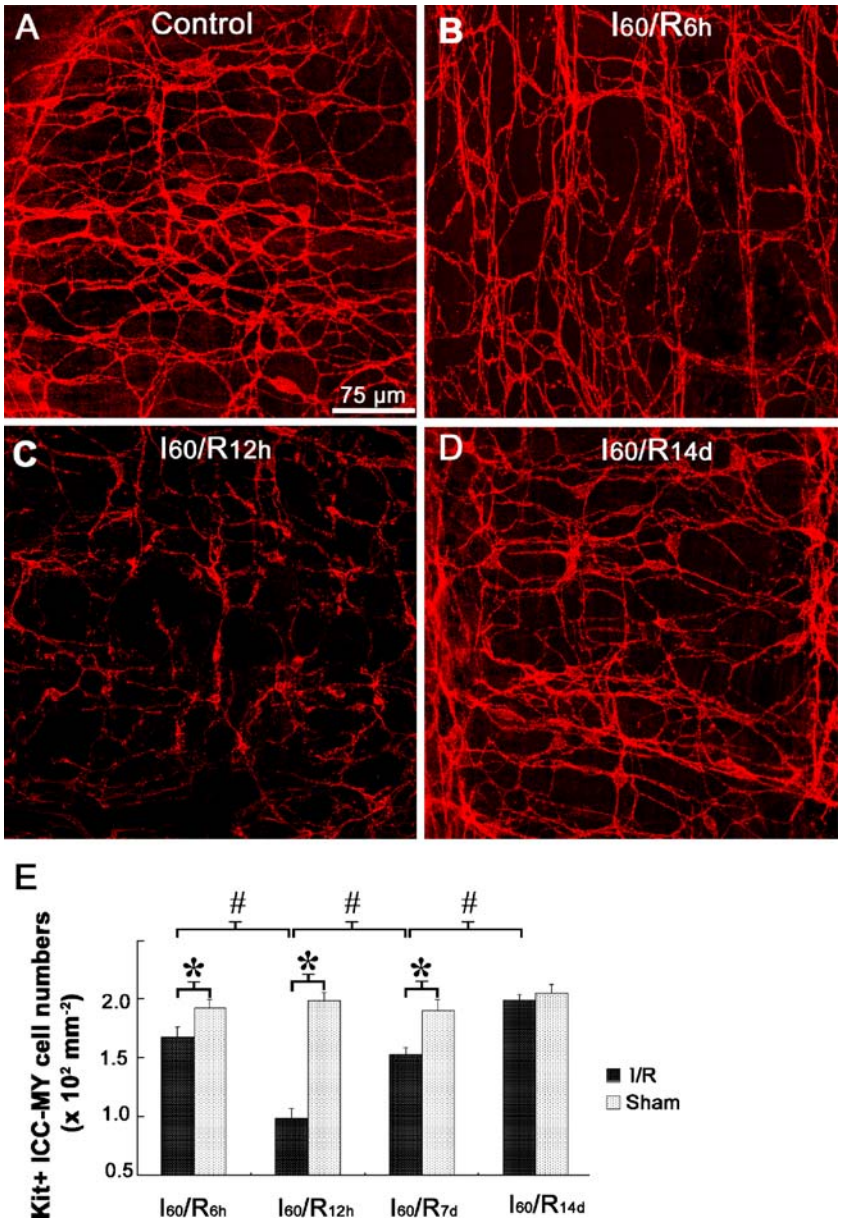

Fig. 1 Confocal images of ICC-MY labeled with ACK2 (*red*) on whole-mount preparations of the small intestine showing the alterations of ICC in the intestinal I/R injury model (**a**–**d**). **a** ICC project their cytoplasmic processes to form an intact cellular network in the control tissue. **b** The Kit+ cells are almost intact after reperfusion for 6 h (I_{60}/R_{6h}; **b**) and these cells are obviously reduced following I_{60}/R_{12h} injury (**c**). **d** A dramatic recovery of ICC is seen in the I_{60}/R_{14d} injury group. **e** The graphs summarized the changes in mean (± SEM) Kit+ ICC-MY cell numbers after I/R insult in guinea pigs. *$P<$ 0.05 indicates significant differences between control and I/R group (n=3; SNK), and #$P<$ 0.05 (SNK) represents significant differences between I/R groups (n=3). *Scale bar* **a** refers to all panels

after I/R to each group of experimental animals. The animals were killed and specimens were stained for ACK2 (Kit) as described above, and then the whole-mount preparations labeled for BrdU as follows: The specimens were additionally fixed in 4% paraformaldehyde in 0.1 M PB at pH 7.2 for 30 min. After rinsing in PBS, the specimens were treated with 2 N HCl for 30 min at 37°C for partial denaturalization of double-stranded DNA. To reveal BrdU, the specimens were incubated with a mouse monoclonal antibody raised against BrdU (3 μg ml^{-1}; DAKO) overnight at 4°C and then a FITC-conjugated secondary antibody (antimouse IgG, 1:100; DAKO).

Measurement and statistical analysis

Photographs of either Kit-positive cells, Kit/TUNEL, or Kit/BrdU double-labeled cells were taken in ten random 0.2607-mm^2 fields (×200 magnification) per whole-mount preparation with a digital camera (SPOT, Diagnostic Instruments, Inc) mounted on a BX51 fluorescence micro-

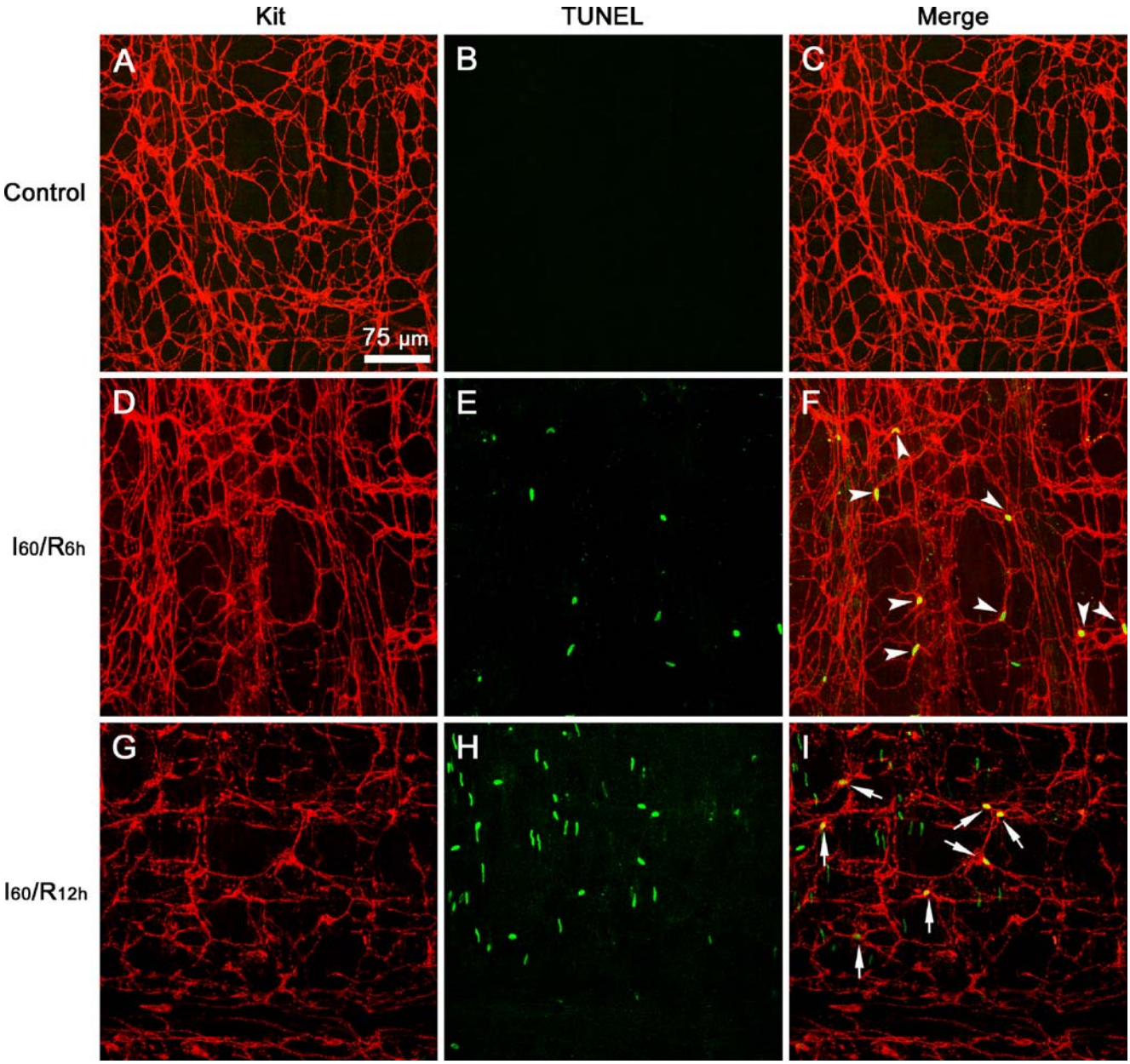

Fig. 2 Confocal photomicrographs showing presumptive apoptotic ICC on whole-mount preparations from the ileum after I/R insult (**a**–**i**). **a**–**c** Kit (*red*)/TUNEL (*green*) double labeling shows that no apoptotic cell is observed in the ICC-MY of control group. **d**–**f** The Kit+(*red*) ICC-MY are almost intact in the I_{60}/R_{6h} group; however, a number of Kit/TUNEL(*green*) double labeling ICC-like cells are visible (*arrowheads*). **g**–**i** The Kit+ ICC-MY networks (*red*) are incomplete following I_{60}/R_{12h} injury with a number of apoptotic ICC (*arrows*). Scale bar **a** refers to all panels

scope (OLYMPUS, Japan). The cell numbers were counted with Image-Pro Plus 5.0 (Media Cybernetics). Five intestinal segments from each experimental animal were sampled for immunofluorescent staining. Data were expressed as means±standard error of the mean (SEM). The *n* value reported in the text refers to the number of animals used. Differences in the data were evaluated by one-way analysis of variance followed by a Student–Newman–Keul's (SNK) post hoc test and $P \leq 0.05$ was taken as a statistically significant difference.

Results

Changes in ICC morphology and density

ICC-MY were located between the longitudinal and circular smooth muscle layers and around the myenteric plexus of the small intestinal wall; cells were revealed by Kit immunohistochemistry in confocal whole-mount preparations (Fig. 1a) that clearly showed ICC processes and numerous branches forming a cellular network in the control. In the I_{60}/R_{6h} group, the density of Kit+ ICC-MY slightly decreased (167.5±8.4 mm^{-2}), and an almost intact cellular networks could be observed (Fig. 1b, e). In the I_{60}/R_{12h} group the density of Kit+ cell numbers were significantly reduced by ~50% compared with the control values (97.6±8.6 mm^{-2}) along with a reduction in the density and thickness of the cellular processes (Fig. 1c, e). Kit+ cell density had partially recovered after 7 days of reperfusion but was still significantly lower than control cell numbers (152.2±6.4 mm^{-2}; Fig. 1e). However, Kit+ cell numbers had recovered to control values and also the cellular networks had restored after 14 days reperfusion (I_{60}/R_{14d} injury; Fig. 1d, e).

Detection of the apoptosis

Apoptosis was detected by the TUNEL method. In control preparations, no cell was labeled by TUNEL method in the intestinal wall on whole-mount preparations (Fig. 2a–c). In the I_{60}/R_{6h} group, the Kit+ ICC-MY networks seemed to be intact; however, a number of Kit+/TUNEL+ (16.7± 3.4 mm^{-2}) were observed which located within the ICC-MY which had identical features with normal ones (Fig. 2d–f; Table 1). The Kit+ cellular networks were incomplete in the I_{60}/R_{12h} injury group, and a number of apoptotic Kit+ ICC were observed (17.4±4.2 mm^{-2}; Fig. 2g–i; Table 1). Kit/vimentin/TUNEL triple labeling showed that those Kit+/TUNEL+ cells were labeled by vimentin in ICC-MY. Some of these cells had long branching processes within ICC-MY (Fig. 3a–d) which were similar in features with normal mature cells, while others had shortened processes with fewer branches (Fig. 3e–h) and probably represented different stages of apoptosis, i.e., those cells with shorter processes were at later stages of apoptosis. In addition, a number of β-tublin III+ enteric neurons which distributed within the myenteric plexus (Fig. 4a–c) and a small number of α-SMA-labeled SMC (Fig. 4d–f) were also marked by TUNEL which hinted that the apoptosis of SMC and enteric neurons were also involved. The quantitative data showed that there were about 17–18 mm^{-2} apoptotic Kit+ cells found in the ICC-MY in either I_{60}/R_{6h} or I_{60}/R_{12h} group (Table 1), and about 10% SMC and 7% enteric neurons were also labeled by TUNEL which suggested that the apoptosis of SMC and enteric neurons was also involved in the I/R injury.

Proliferation of ICC

We used double immunofluorescent staining with anti-Kit/BrdU to reveal the presence of ICC proliferation. Kit/BrdU ICC-MY-like cell labeling was neither seen in the control tissue nor was present 6 and 12 h after reperfusion. However, proliferative cells were frequently seen at 7 days (10.6± 1.1 mm^{-2}) and represented ~5% of the population. This figure was increased by ~2.5 times in the I_{60}/R_{14d} injury group (25.7±4.1 mm^{-2}; Table 1). Cell counting showed that the BrdU+ cells in the ICC-MY represented ~13% of the total Kit-positive cells at the level of myenteric plexus in the I_{60}/R_{14d} injury group. The distribution in many cases was characterized by pairs of cell bodies with processes, and single isolated cells were also observed (Fig. 5a–c). In ICC-DMP, the Kit/BrdU double-labeled cells were often distrib-

Table 1 Mean density of Kit+/TUNEL+ and Kit+/BrdU+ cells in the ICC-MY in the intestinal I/R injury guinea pig model

	I_{60}/R_{6h}	I_{60}/R_{12h}	I_{60}/R_{7d}	I_{60}/R_{14d}
	I/R	I/R	I/R	I/R
Kit+/TUNEL+ cells (mm^{-2}; N=3)	16.7±3.4	17.4±4.2	0*	0
Kit+/BrdU+ cells (mm^{-2}; N=3)	0	0	10.6±1.1*	25.7±4.1

Neither $Kit^+/TUNEL^+$ nor $Kit^+/BrdU^+$ cell was found in control. Data given as mean ± SEM.
N numbers of animals used
*$P<0.05$ (SNK) significantly different to earlier time point (e.g., I_{60}/R_{7d} vs. I_{60}/R_{12h})

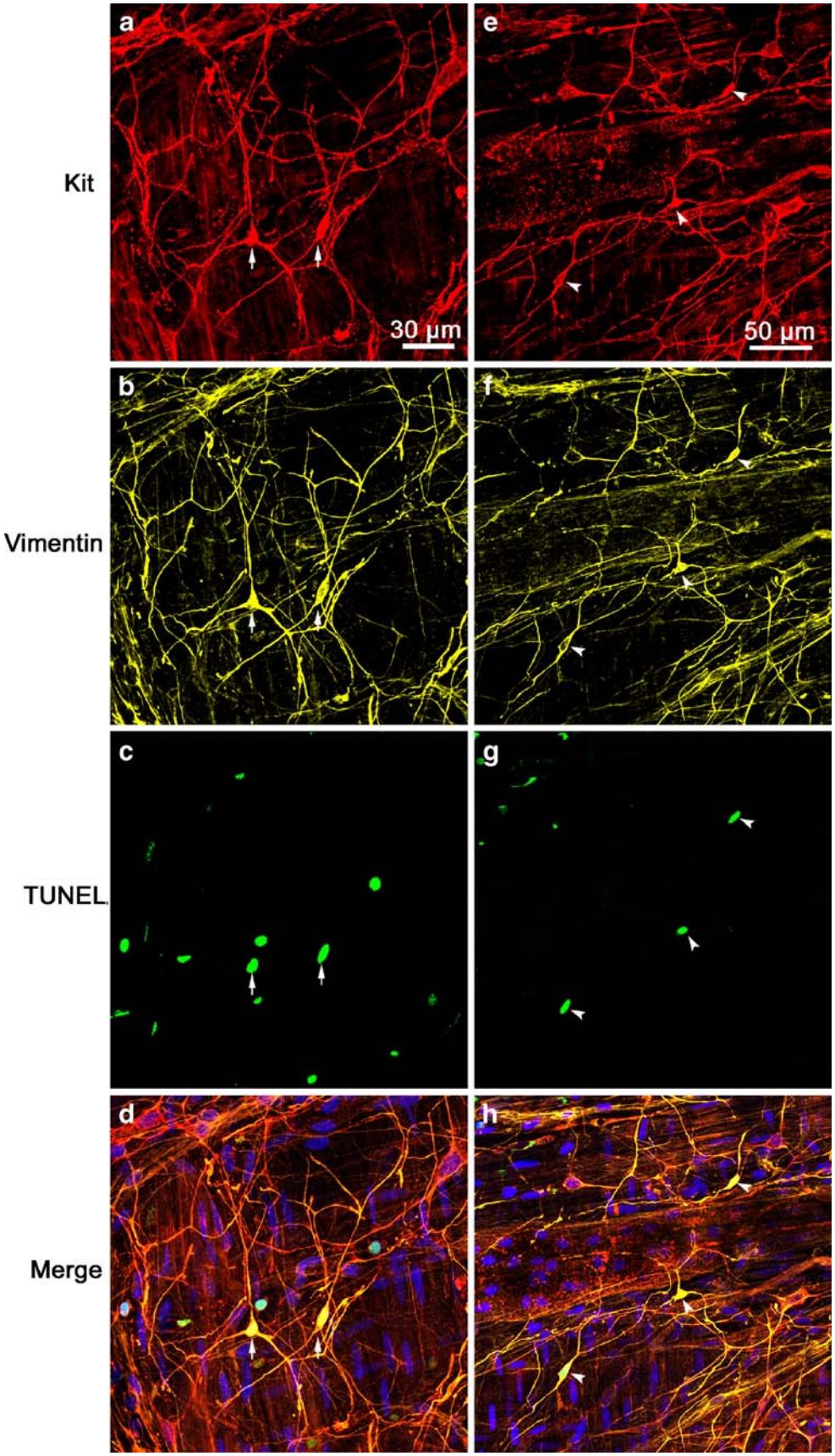

Fig. 3 Confocal images of ICC-MY labeled with Kit (*red*), vimentin (*yellow*), and TUNEL (*green*) on whole-mount preparations of the ileum counterstained with DAPI (*blue*) in the I_{60}/R_{12h} injury group (**a**–**h**). **a**–**d** A few Kit-/vimentin-positive cells are visible with long processes with branches and are labeled by TUNEL (*arrows*). **e**–**h** Tissue is shown at lower magnification that some of the Kit-/vimentin-/TUNEL-positive cells have more shortened processes with fewer branches (*arrowheads*) suggesting a later stage of apoptosis. *Scale bar*=30 (**a**–**d**) and 50 μm (**e**–**h**)

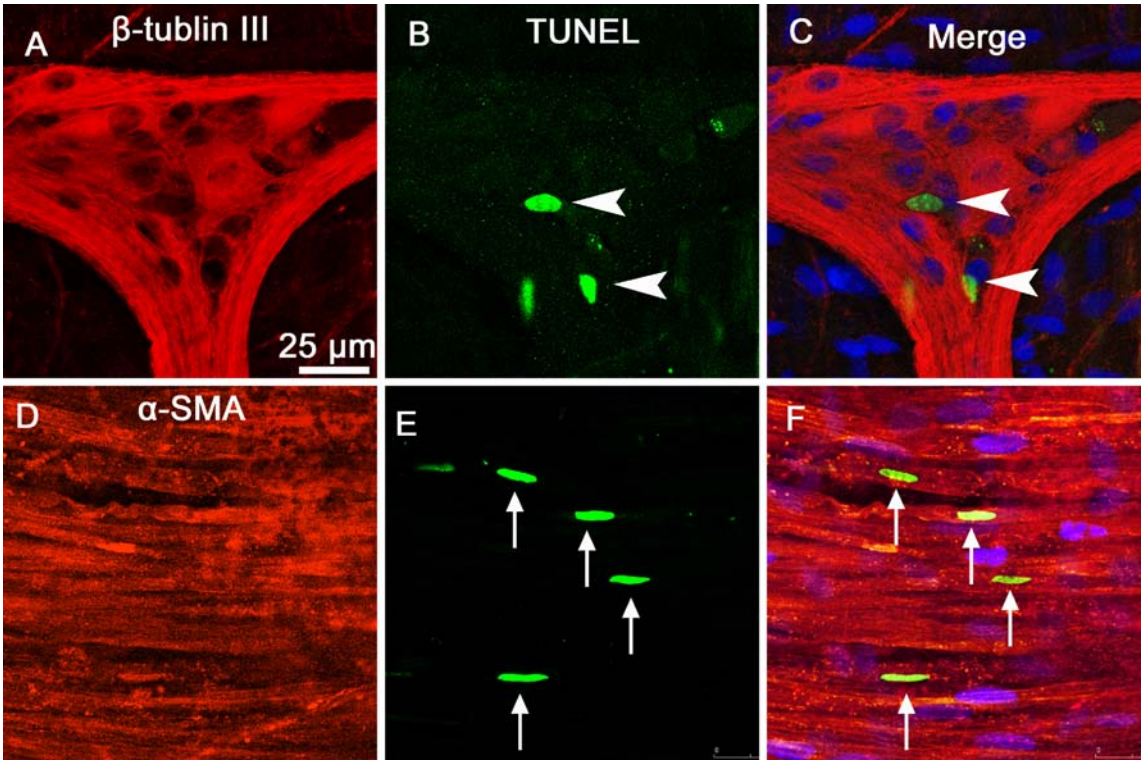

Fig. 4 Confocal photomicrographs showing the TUNEL-positive enteric neurons (**a**–**c**) and smooth muscle cells (**d**–**f**) labeled by anti-β-tublin III and anti-α-SMA antibodies respectively on whole-mount preparations of the ileum counterstained with DAPI (*blue*) from I_{60}/R_{12h} injury animals. **a**–**c** Several β-tublin III (*red*) and TUNEL (*green*)-double-positive cells (*arrowheads*) are present. Note, β-tublin III immunoreactivity does not stain nuclei, thus is clearly differentiated from the nuclear TUNEL label. Cells were also present after double labeling (*arrows*) with α-SMA (*red*) and TUNEL (*green*; **d**–**f**). *Scale bar* **a** refers to all panels

uted in pairs (Fig. 5d–f), and they had relatively round cell bodies and long slender processes which could be identified as ICC-DMP according to their features, although less and thinner processes were seen comparing with mature ones.

Discussion

The present study demonstrates that (1) the Kit-positive ICC numbers are reduced after the intestinal I/R injury but make a dramatic recovery; (2) the I/R injury can lead to the apoptosis of ICC, SMC, and enteric neurons; (3) proliferation of ICC is involved in the recovery of the ICC. It has been reported that I/R injury can lead to gastrointestinal dysfunctions, such as a delay in gastrointestinal transit [3, 4], alterations of the migrating motor complex [5], and alterations in in vivo motor responsiveness to pharmacological stimulation [6]. In addition, in vitro experiments reveal a reduction in mechanical contraction of the smooth muscle layers [1], although the underlying mechanisms are not clearly understood. In partial agreement with the present data, a rat intestinal I/R injury model has revealed that a loss of Kit+ ICC is concomitant with the weakening of spontaneous mechanical contractions, presumably driven by ICC, which subsequently recover to normal as well as ICC Kit labeling. On this basis, it has been suggested that ICC play a central role in I/R-caused motility disorders [2]. In support of this hypothesis, our results also show a 50% decrease of Kit+ cell numbers in ICC-MY in the intestinal I/R guinea pig model. A loss and reappearance of Kit expression maybe one of the underlying mechanisms associated with motility disorders such as that has been suggested by intestinal obstruction [19] and surgical resection models [18]. Others have postulated that the loss of Kit may lead to transdifferentiation of ICC toward a smooth muscle cell phenotype on the basis of Kit signaling blockade experiments in the neonatal mice with an anti-Kit antibody [27]. Therefore, we have stained our tissue using anti-Kit and anti-α-SMA (a special marker of SMC) for cells in a transdifferentiation stage where both proteins might be present. Double-labeled cell is not seen and thus, it seems not to support a case for ICC transdifferentiation toward a SMC phenotype under our experimental conditions (supplemental Fig. 2A–C). However, as α-SMA is not an established marker for those transdifferentiated cells, a further observation under electron microscopy would be necessary to exclude or include this possibility.

Is the switch of Kit expression the only explanation for the alteration of Kit+ ICC as suggested in a rat model of I/R injury? It should be noted that in animal models of I/R injuries

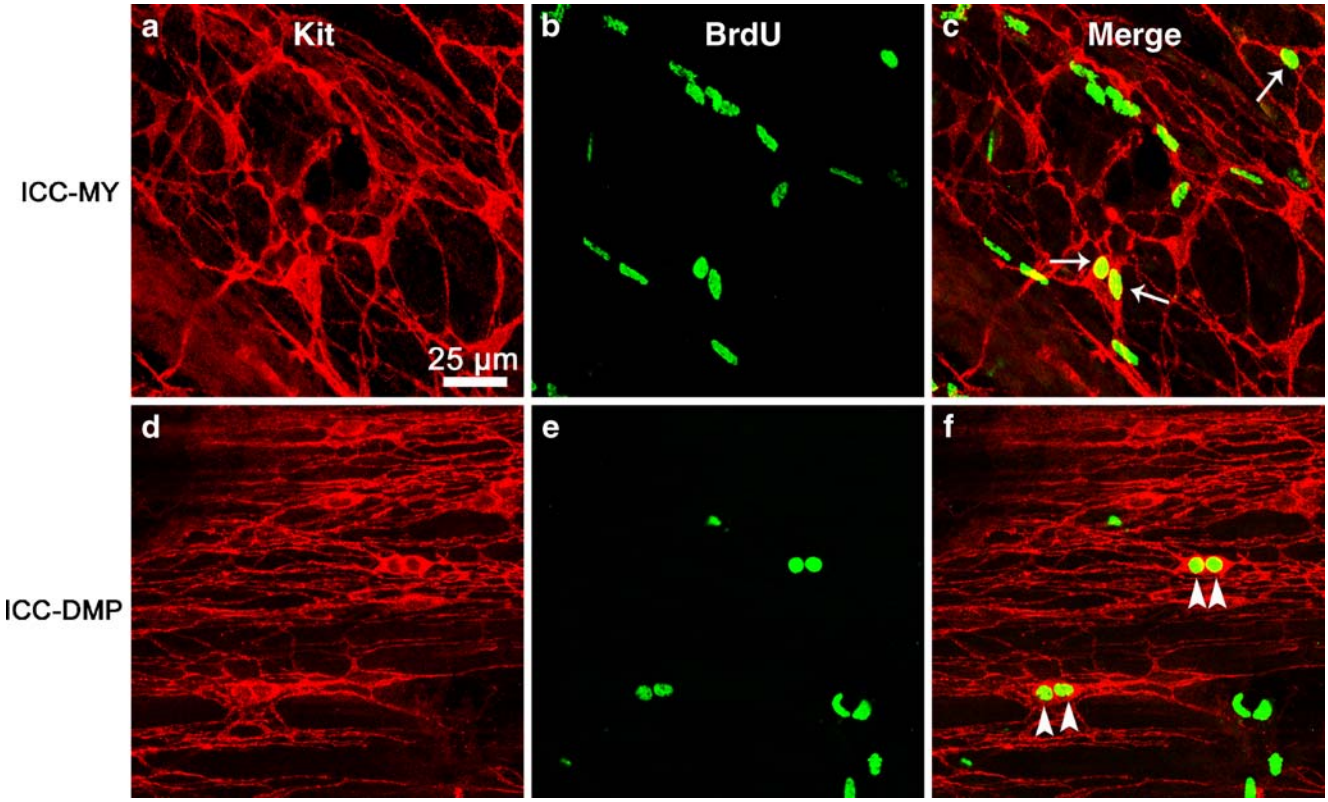

Fig. 5 Confocal photomicrographs showing presumptive proliferating ICC on whole-mount preparations from the ileum in I_{60}/R_{14d} injury group. **a**–**c** A number of double labeled of Kit (*red*) and BrdU (*green*) cells (*arrows*) that have processes with branches are present in the ICC-MY and resemble normal mature cells. **d**–**f** The Kit(+)/BrdU(+) cells often distribute in pairs in ICC-DMP, with closely adjacent cell bodies (*arrowheads*). *Scale bar* **a** refers to all panels

to the liver [21], kidney [22], heart [28], and lung [24], as well as intestine [23], apoptosis is often present in the tissues. We have confirmed these previous results showing cell death by using TUNEL methodology and about 10–15% of the apoptotic Kit+ cells are at an early stage after I/R. Vimentin, structural protein, is colocalized with Kit in ICC [26]. The Kit/vimentin/TUNEL labeling cells further confirm a true loss of ICC which suggests that the apoptosis of ICC is also involved in the cell number reduction as well as the reduction of Kit expression. Besides that, it should be noted that apoptotic ICC might cease Kit expression at certain stage; therefore, those Kit+/TUNEL+ cells may only represent a part of apoptotic ICC. In addition, a number of SMC and enteric neurons are also labeled by TUNEL. Therefore, a conclusion is made that the intestinal motility dysfunctions that caused by I/R injury could be interpreted as a compound result of both the Kit expression reduction and the apoptosis of ICC, SMC, and enteric neurons.

Our findings confirm, by using BrdU incorporation, that there is a restoration of the Kit+ ICC cell numbers in both ICC-MY and ICC-DMP, at least in part, via proliferation for at least 7 days after an I/R insult in the adult guinea pig. We do not find out the presence of cell proliferation (Kit/BrdU double-labeled ICC-like cells) in the control tissue over a similar time period. Similarly, our recent findings also have indicated that an age-dependent proliferation is involved in the expansion of ICC cell number in the murine small intestine aged from postpartum days 0 to 16, and the proliferation vanishes in adult mice [29], and under in vitro culture conditions, only ICC from neonatal mice before day 6 postpartum can proliferate in response to Kit ligand which also suggests a time-limited proliferation of ICC [30]. Therefore, it seems that ICC or ICC progenitor do not proliferate generally in adult animals as shown in our previous work [25].

The present study has shown that proliferation takes a part in the restoration of ICC after loss. One question is what kind of causes probably promote the proliferation? ICC are actually reduced in number by apoptosis and this suggests that the recovery of ICC is due at least in part to generation of new ICC. A number of reduced ICC may cease expression of Kit and change into a kind of intermediate fibroblast-like cells after insult and, in all likelihood, only Kit expression resumption is involved in their recovery.

Another question is what kind of cells may have given rise to the proliferation? A recent study has successfully identified a very small group of $Kit^+/CD44^+/CD34^+$/insulin $receptor^+$/insulin-like growth factor I receptor $(IGF\text{-}IR)^+$ cells in murine stomach as the putative progenitors of ICC

which could be expanded under the stimulation of Kit ligand, stem cell factor, and further transdifferentiate into mature ones [31], and similarly, our recent study has also shown that a number of Kit+/BrdU+ cells (new-emerged ICC) which appear during postnatal development in the murine small intestine are also labeled with anti-CD44, CD34, and IGF-IR antibodies. Therefore, it is likely that those new-emerged ICC (Brdu+/Kit+ cells) after ICC loss in adult animals originate from the proliferation and differentiation of progenitor cells in this case, although this hypothesis needs to be proved by further investigations.

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Conflict of interest statement We declare that we have no conflict of interest.

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